Adjusting serum urate level by affecting membrane transporters involved in the disposition of urate

輸送体を介した血清尿酸値調節

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Abbreviations

AhR	aryl hydrocarbon receptor
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein
C/M ratio	cell-to-medium ratio
cRNA	complimentary RNA
D-MEM	Dulbecco' s modified Eagle's medium
FBS	fetal bovine serum
FITC	Fluorescein isothiocyanate
HPRT1	hypoxanthine guaninephosphoribosyltransferase 1
IC_{50}	half-maximum inhibition concentration
IS	indoxyl sulfate
K_m	Michaelis-Menten constant
MBS	Modified Barth's solution
3-MC	3-methylchoranthrene
MRP4	multidrug resistance-associated protein 4
MSU	monosodium urate
NPT	sodium phosphate transporter
OAT	organic anion transporter
P_{app}	apparent permeability coefficient
PBS	phosphate buffered saline

PhA	pheophorbide a
PPARα	peroxisome proliferator-activated receptor-α
SMCT	sodium-coupled monocarboxylate transporter
SUA	serum urate level
UOX1	urate oxidase
URAT1	urate transporter 1
URATv1	voltage-driven urate transporter 1

Chapter 1 Introduction

Urate is a weak organic acid with a pKa value of 5.8. It is the end product of purine degradation in humans and some higher primates. Due to the mutation of urate oxidase gene occurred in Miocene epoch [1-3], urate in humans cannot undergo further oxidation catalyzed by urate oxidase (UOX1 or uricase) to form allantoin, a more water-soluble substance with being easily excreted. Loss of urate oxidase results in higher serum urate level (SUA) in humans, compared with those in other mammals [4-5]. Mutation of urate oxidase and increased serum urate level were believed to be of critical importance in creating human by working as a cerebral stimulant to accelerate brain development and improve human intelligence because of the similarities of urate to other cerebral stimulants, such as caffeine or theobromine [6]. Urate also has strong antioxidant properties and helps humans survive in the oxygen environment by cleaning oxidants, such as singlet oxygen and hydroperoxyl radicals, which are harmful to humans. It is reported that urate eliminates about as much as 60% of free radicals in human serum [7-9]. Increased serum urate level owing to evolutionary loss of uricase in humans is regarded as compensation to the mutation of L-gulonolactone oxidase, which is responsible for the synthesis of another important antioxidant substance, vitamin C [10-13]. In addition to its antioxidant function, urate seems to play a pivotal role in the maintenance of *in vivo* blood pressure homeostasis in humans [14, 15].

Epidemiologic studies show that abnormal serum urate level is associated with several diseases. For example, hyperuricemia is closely related to gout attack, a disease with a history of more than 5,000 years ever since its first documentation in Egypt [16]. Gout is featured by the accumulation of monosodium urate (MSU) monohydrate crystals in joints [17-20]. Urate crystals deposited in joint lead to inflammation responsive via activating NLRP3 inflammasome [20]. Hyperuricemia (serum urate level over 420 μM for

men, over 360 μM for women) may increase the risk of gout attack as reported by many studies [21-24]. Previous reports also indicate that hyperuricemia might play a role in the development of coronary heart disease, stroke, hypertension, diabetes mellitus, renal diseases and other cardiovascular diseases [25-42]. Because urate has anti-oxidant property, reduced SUA level may cause harmful effect in humans. Reduced serum urate level may decrease the antioxidant ability of humans. Indeed, hypouricemia (serum urate level lower than 120 μM) has already been linked to Hodgkin's disease and Alzheimer's disease [43-46]. Thus, maintenance of normal serum urate level is crucial to human health. Due to the close relationship of urate with a number of diseases, it has been used as a biomarker for many diseases [47-50] and is now involved in regular clinical blood test. Clinical and animal studies have suggested the participation of urate in the generation and development of diseases, and the importance of controlling urate level as a preventing and/or treating method of such diseases as well [45].

Generation of urate primarily occurs in liver, muscles and intestine, while excretion of urate mainly occurs from the kidney and intestine [51-52]. Of the urate daily produced, about two thirds is excreted from kidney and the rest is mainly eliminated directly across intestinal epithelial cells from blood [52-53]. In kidney, there is a urate transport system located on renal proximal tubule which plays an important role in the regulation of serum urate level. Most of urate is filtrated at the glomerulus and reabsorbed by this transport system. In 2002, Enomoto *et al*. identified human urate transporter 1 (URAT1, encoded by *SLC22A12*) as a reabsorptive urate transporter on the apical membrane of renal proximal tubule where it plays a predominant role in urate uptake from urine [54]. Mutations and defects of human URAT1 have been reported to result in hypouricemia [55-57]. Previous studies found that many drugs (such as benzbromarone and losartan) could decrease serum urate level by

exhibiting inhibitory effects on URAT1 [58-60]. These studies have suggested an essential role of URAT1 in the transport of urate.

Similarly, organic onion transporter 4 (OAT4, encoded by SLC22A11) and OAT10, encoded by SLC22A13, are highly expressed at the apical side of proximal tubular cells and also involved in the reabsorptive transport of urate from luminal side into tubular cells [61-63]. On the basolateral membranes of proximal tubular cell, a voltage-driven urate transporter, URATv1 (GLUT9) encoded by SLC2A9 gene is recently reported as a solute carrier responsible for the urate transport from the tubular cells into blood [64]. Hypouricemia was also found in patients with the loss-of-function mutations in URATv1, independent of genetics of URAT1 [64]. URAT1, OAT4, and OAT10 at the apical side of renal proximal tubule and URATv1 at the basolateral side of proximal tubule cells together consist of the vectorial transport form the urine to blood (as is shown in **Fig. 1-1**).

Since urate simultaneously undergoes secretion from blood to urine, other transporter system may also important to consider renal handling of urate in kidney. The system is possibly consisted of organic onion transporter 1 (OAT1, encoded by SLC22A6), and organic onion transporter 3 (OAT1, encoded by SLC22A8) on the basolateral side of proximal tubule cells, and sodium phosphate transporter 1 (NPT1, encoded by SLC17A1), NPT4 (encoded by SLC17A3), multidrug resistance protein 4 (MRP4, encoded by ABCC4), and breast cancer resistance protein (BCRP, encoded by ABCG2) [65-74].

Serum urate level is also influenced by non-renal urate transport pathway. In humans, expression of BCRP in kidney is relatively low compared with other organs [73]. On the contrary, expression of BCRP in intestine is high where one third of urate daily generated is excreted, compared with the expression in kidney. BCRP is expressed at the apical side of intestinal epithelial cells and

mediates the urate efflux transport across the intestinal epithelial wall. Reduction of BCRP function is closely related to gout and hyperuricemia as is demonstrated by recent genome-wide association studies [75-76]. Thus, BCRP can be regarded as an important efflux transporter mediating the non-renal excretion of urate (as is shown in **Fig. 1-2**).

With the development of economy, and westernization of lifestyle, the past several decades have witnessed an obvious increase in the prevalence of diseases such as cardiovascular disease, obesity, hyperuricemia, diabetes mellitus, which are called “rich man’s diseases” and the prevalence of these disease will continue to increase in the following several decades [77-79]. Serum urate level seems to be closely associated with these diseases [80]. Due to the important role of urate transporters in regulation serum urate level, modulation of these urate transporters to control serum urate level is of critical significance. The present thesis will focus on this topic and investigate the modulation of urate transporters to affect serum urate level.

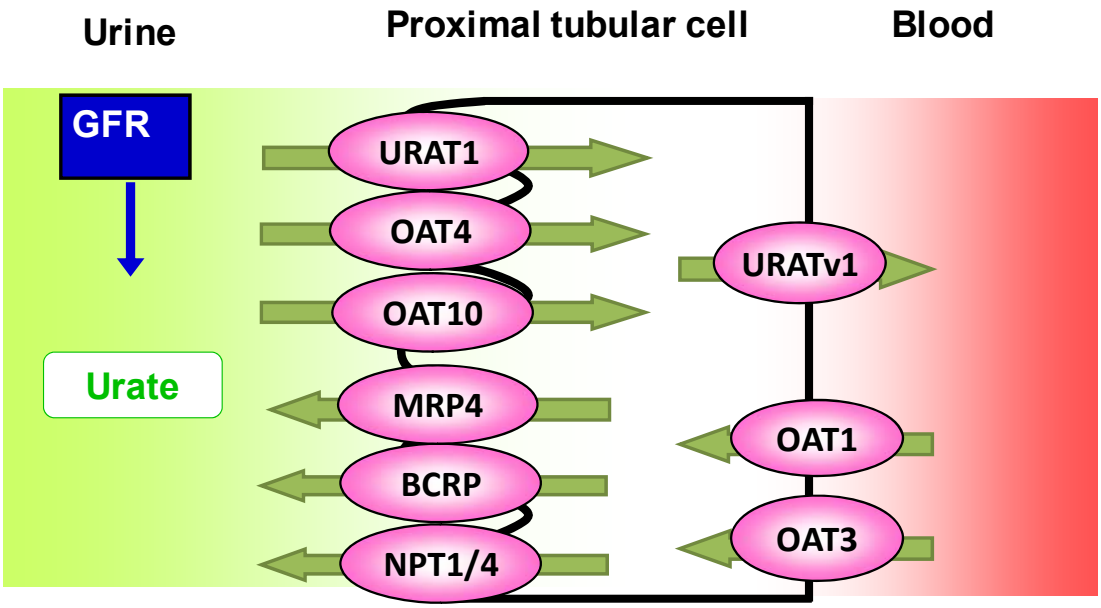

Fig. 1-1 Transporter mediated urate transport in renal proximal tubule. On the apical side of renal proximal tubule, URAT1, OAT4, and OAT10 are responsible for the reabsorptive transport of urate from luminal side into renal

proximal tubular cell. On the basolateral side, URATv1 is responsible for the transport of urate from proximal tubular cell into blood. OAT1 and OAT3 on the basolateral side mediate the urate excretory transport from blood into proximal tubular cell. MRP4, BCRP, NPT1 and NPT4 located on the apical side functions as excretory transporter and transport urate from proximal tubular cell into urine.

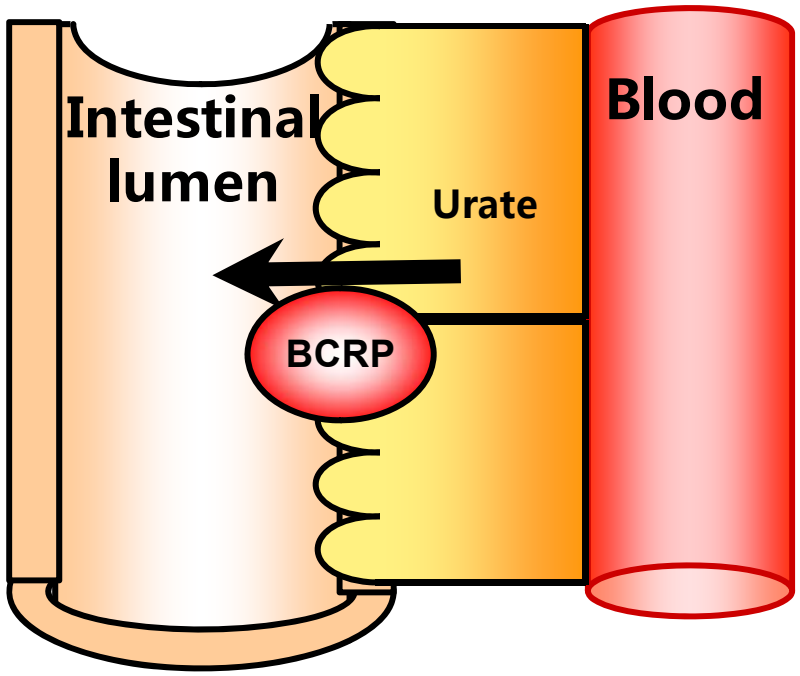

Fig. 1-2 Transporter mediated urate transport in intestine. BCRP is located at the apical side of intestinal epithelial cells and responsible for the urate efflux transport into intestinal lumen.

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Chapter 2 Functional cooperation of SMCTs and URAT1 for renal reabsorption transport of urate

Abstract:

Urate is mainly excreted into urine in humans. Serum urate level is regulated by a urate transport system located on renal proximal tubule. Urate transporter 1 (URAT1) is located on the apical side of renal proximal tubule and is responsible for the reabsorption of urate from luminal side into tubular cells. At the same site, it has been hypothesized that sodium-coupled monocarboxylate transporters (SMCTs) are responsible for the transportation of monocarboxylates such as lactate and nicotinate, which are exchanged for urate transport via URAT1 as the driving force. Accordingly, SMCTs could indirectly stimulate URAT1-mediated urate reabsorption by providing a counter ion, monocarboxylates, for the exchange.

The present study investigated to clarify the hypothesized functional cooperative relationship between URAT1 and SMCTs in the reabsorptive transport of urate. By preloading nicotinate in SMCT1-URAT1 co-expressing *Xenopus* oocytes, URAT1-mediated urate transport was stimulated by preloading nicotinate. Nicotinate was taken up by SMCT1 but not by URAT1. When removing sodium ion from the uptake medium, the stimulation effect was decreased. When adding SMCT1 inhibitors, the stimulation effect was also reduced. The results from this study indicate the cooperative relationship of URAT1 and SMCT1, and that SMCT1 is a potential target for the alteration of renal handling of urate indirectly.

2-1 Introduction

Renal urate transport system is essential to the regulation of serum urate level. In kidney, many urate transporters including URAT1 are involved in renal handling of urate. Monocarboxylates are the counterpart of urate in the transport of urate via URAT1. Recently, two members of sodium-coupled monocarboxylate transporters were identified and characterized as monocarboxylate transporters with electrogenic nature, in which SMCT1 and SMCT2 were encoded by *SLC5A8* and *SLC5A12*, respectively [81-82]. Substrates of SMCT1 and SMCT2 include lactate, nicotinate, and butyrate [81-82]. Both of them are reported to locate on the apical side of proximal tubular cells [81-82]. SMCTs are involved in the absorption of monocarboxylates in a sodium-dependent manner, and it is hypothesized that SMCTs enhance URAT1-mediated urate reabsorption by providing monocarboxylates for the exchange transport with urate. Moreover, Thangaraju *et al*. observed the decrease of serum urate level and increase of urinary excretion of urate in mice that are knocked out of both *Slc5a8* and *Slc5a12* in the kidney [83]. Serum lactate level was also reduced in the knockout mice. These observations indicated a possible role of SMCT1 and SMCT2 in the reabsorptive transport of urate in kidney. Accordingly, it is considered that URAT1 and SMCTs are linked via lactate and/or other monocarboxylates transport [84-86]. Furthermore, it has been reported that PDZK1, which is a PDZ domain containing protein located on the renal proximal tubule, could bind to URAT1 at the C terminal part of URAT1 [87]. In addition, SMCT1 and SMCT2 were reported to be binding partners of PDZK1 [84]. These findings suggested possible physiological links between URAT1 and SMCTs in the renal reabsorption of urate in renal proximal tubule. However, to date there is no direct functional evidence to show the cooperative relationship of URAT1 and SMCTs in urate transport. Collectively, it is thought that SMCTs and URAT1 have a cooperative relationship in urate transport; therefore, we designed the current study and investigated the cooperative

relationship between SMCTs and URAT1 using the *Xenopus* oocytes gene-expressing system. The results of this study provide strong support for the functional links of SMCTs and URAT1 in the transport of urate.

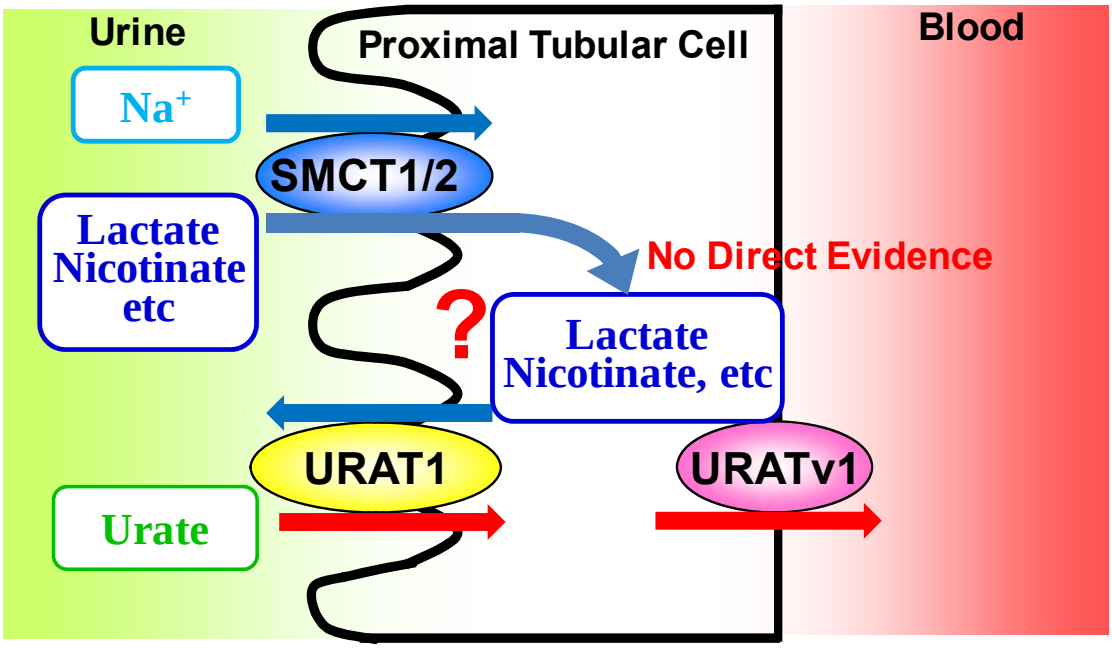


Fig. 2-1 Hypothesized urate transport model at renal proximal tubule. SMCTs take up monocarboxylates, such as lactate and nicotinate, from luminal side into proximal tubular cells. The monocarboxylates taken up by SMCTs then exchange with urate via URAT1 enhancing URAT1-mediated urate reabsorptive transport.

2-2 Materials and Methods

2-2-1 Chemicals and reagents

[^{14}C]Urate (1.96 TBq/mol) was obtained from Moravek Biochemicals, Inc. (Brea, CA). [^{3}H]Nicotinate (37 GBq/mol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Nicotinate (purity>98%), collagenase and gentamicin sulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium L-lactate (purity around 98%), sodium butyrate (purity>98.5%), N-methyl-D-glucamine (NMDG) and phenol/chloroform/isoamyl alcohol (25:24:1) were the products of Sigma-Aldrich (St. Louis, MO). Clearsol-I was obtained from Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade.

2-2-2 Preparation of human URAT1 and SMCT1 cRNA

Human URAT1 plasmid DNA, which was cloned in pGEMHE vector, was synthesized according to the method previously described [88] and then the plasmid DNA was digested with *Nhe*I (Takara Bio Inc., Otsu, Japan) before cRNA preparation. SMCT1 plasmid DNA cloned in pGH19 vector [81] was kindly provided by Professor Seiji Miyauchi at Toho University and was linearized by *Pst*I (Takara Bio Inc., Otsu, Japan). cRNA of URAT1 and SMCT1 were synthesized by *in vitro* transcription method using T7 mMESSAGE-mMACHINE kit (Ambion, Austin, TX). One μg of the linearized template DNA was mixed with 10 μL of 2X NTP/CAP, 2 μL of 10X Reaction Buffer, 2 μL Enzyme Mix supplemented with purified water to 20 μL, and incubated in a water bath maintained at 37 ^{o}C for 2h. Then, 1 μL of TURBO DNase was added to the reaction tube and the tube was incubated at 37 °C for 15 min. After that, 115 μL of pure water and 15 μL of ammonium acetate stop solution were added, and mixed thoroughly. 150 μL of phenol/chloroform/ isoamyl alcohol (25:24:1) was added to extract RNA. After centrifuged at 15,000 rpm for 15 min (4 °C), the aqueous phase (upper phase) of the sample was transferred into another tube. Sample was then added with an equal

volume of chloroform, vortexed for 2min, and centrifuged at 15,000 rpm for 15 min (4 °C). The aqueous phase (upper phase) of the sample was transferred into another tube. RNA was precipitated by the addition of an equal volume of isopropanol and chilled at –20 °C overnight. Pellet of RNA was obtained by centrifuging the sample at 15,000 rpm for 20 min (4 °C). RNA pellet was then washed by the addition of 100 µL of 70% ethyl alcohol and dried in a water bath maintained at 37°C for 1 h to evaporate residual ethyl alcohol. RNA was dissolved in purified water. Concentration of RNA was determined by UV method on an Eppeendorf BioPhotometer (Eppendorf, Hamburg, Germany).

2-2-3 Preparation of *Xenopus* oocytes

Xenopus laevis provided by Hamamatsu Biological Research Service, Inc. (Hamamatsu, Japan) were anaesthetized for 30 min in a mixture of ice and water. Then *Xenopus laevis* were put on ice and oocytes were taken out from ovaries of *Xenopus laevis*. After washed by Oocyte Ringer 2 (OR2) solutions 10 times, oocytes were incubated with 2 mg/mL collagenase (dissolved in OR2 solution) for up to 20 min at room temperature. Then oocytes were washed by pH7.4 OR2 solution another 10 times and were transferred into a dish containing the modified Barth solution (MBS, pH7.4) supplemented with 50 µg/mL gentamycin. Oocytes were defolliculated with fine forceps under Olympus SZ61 stereo microscope (Olympus Optical Co. Ltd., Tokyo, Japan) before use. Defolliculation of the oocytes was carried out in pH7.4 Defolliculation solution.

Oocyte Ringer 2 (OR2) solution:
82.5 mM NaCl
2 mM KCl
10 mM MgCl2
5 mM HEPES
Adjust pH to 7.4

Modified Barth's solution (MBS):

88 mM NaCl

1 mM KCl

0.33 mM $Ca(NO_3)_2$

0.41 mM $CaCl_2$

0.82 mM $MgSO_4$

2.4 mM $NaHCO_3$

10 mM HEPES

Adjust pH to 7.4.

Defolliculation solution

110 mM NaCl

1 mM EDTA*2Na

10 mM HEPES

Adjust pH to 7.4.

2-2-4 Expression of URAT1 and SMCT1 protein in *Xenopus laevis* oocytes

URAT1 and SMCT1 protein were expressed in *Xenopus* oocytes by microinjection method. In preparing oocytes expressing URAT1 alone, 12.5 ng URAT1 cRNA (50 nL) was injected into each oocyte by a Drummond Digital Microdispenser (Drummond Scientific Company; Broomall, PA, USA). In oocytes expressing both URAT1 and SMCT1, a 50 nL mixture of 12.5 ng URAT1 cRNA and 12.5 ng SMCT1 cRNA was injected into each oocyte. The rest of the oocytes were injected the same volume of water as background. After microinjection, *Xenopus* oocytes were cultured in MBS containing 50 μg/mL gentamycin for 2-3 days at 18 ^{o}C before the uptake experiment. All experiments concerning *Xenopus laevis* were carried out according to the guiding principles promulgated by the Institutional Animal Care and Use

Committee of Kanazawa University.

2-2-5 Uptake study by *Xenopus laevis* oocyte

For urate uptake experiments, oocytes were preloaded with monocarboxylate by preincubation for 60 min or the indicated time in each result at 25 ^{o}C in ND96 buffer in the presence or absence of sodium ions (in the study investigating sodium effect, sodium ions were substituted by NMDG) containing monocarboxylate before urate uptake. Then, the oocytes were washed three times with 25^{o}C ND96 buffer in the presence or absence of sodium ions. The uptake study was carried out for 60 min at 25 ^{o}C in ND96 buffer in the presence or absence of sodium ions containing 10 μM [^{14}C]urate. For nicotinate uptake experiments, the uptake study was carried out at 25 ^{o}C in ND96 buffer containing radio-labeled and unlabeled nicotinate and was stopped by removing the uptake buffer, and then the oocytes were washed three times with ice-cold uptake buffer. Each oocyte was transferred into a microcentrifuge tube containing 50 μL 5% sodium dodecyl sulfate solution. After homogenization of the oocytes, 1.5 mL of Clearsol-I was added into each tube for quantitation of radioactivity.

Uptake buffer used in this study:
pH 7.4 ND96 buffer:
96 mM NaCl
2 mM KCl
1 mM $MgCl_2$
5 mM HEPES
Adjust pH to 7.4.

ND96 buffer free of sodium ions:
192 mM N-methyl-D-glucamine
plus 19.2 mL 5N HCl

3.6 mM $CaCl_2$

4 mM KCl

2 mM $MgCl_2$

10 mM HEPES

Adjust pH to 7.4

2-2-6 Analytical method

A liquid scintillation counter (LSC-5100, Aloka, Tokyo) was used to determine the radioactivity. The amount of [14C]urate or [3H]nicotinate taken up by oocytes was calculated according to the radioactivity obtained from each oocyte. Uptake of urate or nicotinate, expressed as the cell-to-medium (C/M) ratio (μL/oocyte), was calculated by dividing the uptake amount by the concentration of substrate in the uptake buffer. Results were expressed as mean ± SEM. Statistical differences were analyzed by Student's t-test. A *p* value less than 0.05 was considered statistically significant.

2-3 Results

2-3-1 Effect of lactate on URAT1-mediated urate uptake

Initially, urate uptake by oocytes expressing URAT1 alone or SMCT1-URAT1 co-expressing oocytes was determined after preloading 1.5 mM L-lactate for 60 min before urate uptake, respectively. The result is shown in **Fig. 2-2**. Urate uptake by oocytes expressing both SMCT1 and URAT1 was higher than that by oocytes expressing URAT1 alone, as shown in **Fig. 2-2**. The increase of urate uptake can be attributable to two mechanisms. Firstly, the L-lactate taken up by SMCT1 can be high enough to be exchanged with urate and enhance the urate uptake. The second is due to the different expression level of URAT1 between oocytes expressing URAT1 alone and both of SMCT1 and URAT1. The former mechanism, which is a hypothesis of the present study, was further examined.

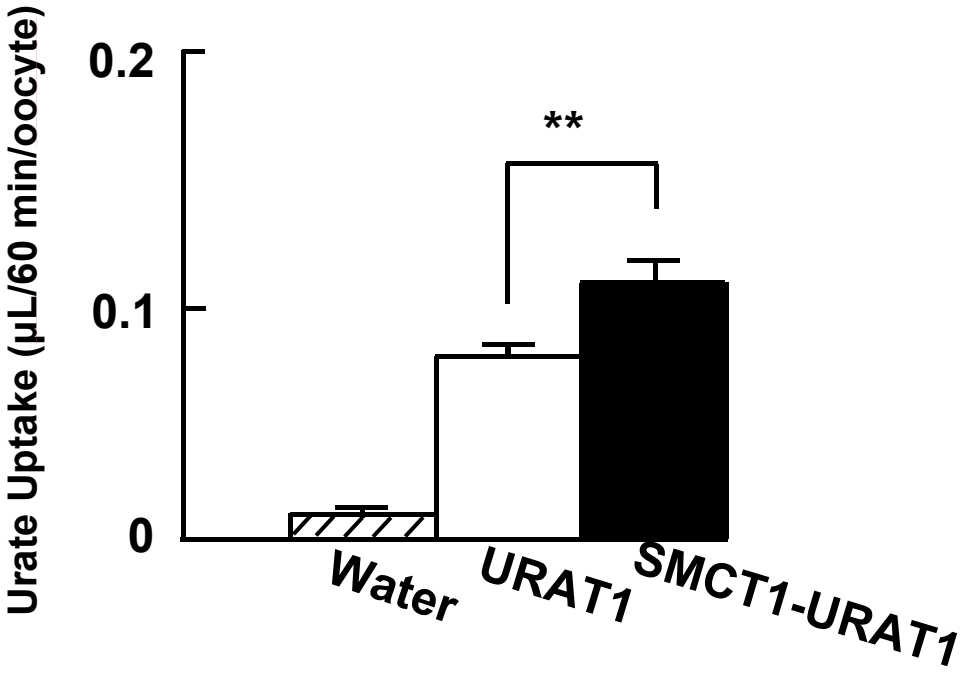


Fig. 2-2 Stimulation effect of monocarboxylate on urate uptake. SMCT-URAT1 co-expressing oocytes (closed bar), oocytes expressing URAT1 alone (open bar) and water-injected oocytes (slashed bar) were preincubated in ND96 buffer (pH7.4) containing 1.5 mM sodium lactate for 60 min. Then, the uptake study was carried out in ND96 buffer 10 μM [^{14}C]urate at pH7.4 for 60

min. Each point represents the mean ± SEM from 9-10 oocytes. Student's t-test: **p<0.01 *vs*. URAT1-mediated urate uptake in oocytes expressing URAT1 alone.

2-3-2 Effect of different monocarboxylates on URAT1-mediated urate uptake in *Xenopus* oocytes expressing both SMCT1 and URAT1

In order to eliminate the differences of uptakes caused by the difference of expression level of transporter proteins between URAT1-alone and SMCT1-URAT1 double expressing oocytes, and to optimize condition for this study, the effect of several other monocarboxylates on urate uptake was examined in SMCT1-URAT1 co-expressing oocytes. Nicotinate, butyrate, and L-lactate were used as monocarboxylates to examine the stimulation effect of urate transport by URAT1. The results are shown in **Fig. 2-3.** SMCT1-URAT1 co-expressing oocytes were preincubated with 1.5 mM nicotinate, butyrate, or L-lactate for 60 min before initiation of urate uptake. Then, uptake of urate by SMCT1-URAT1 co-expressing oocytes was measured for 60 min at 25 ^{o}C in ND96 buffer containing 10 μM [^{14}C]urate. Nicotinate and L-lactate exhibited a stimulation effect on urate uptake. Nicotinate exhibited the highest stimulation effect of the urate uptake, which was as much as 8.7 folds compared with the control (SMCT1-URAT1 co-expressing oocytes without preloading of nicotinate) group. L-Lactate also exhibited a stimulation effect with an increase of 28% compared with control group. As for butyrate, although it tends to stimulate (22%), there was no statistically significant difference compared with control group. Therefore, nicotinate was used in the following studies.

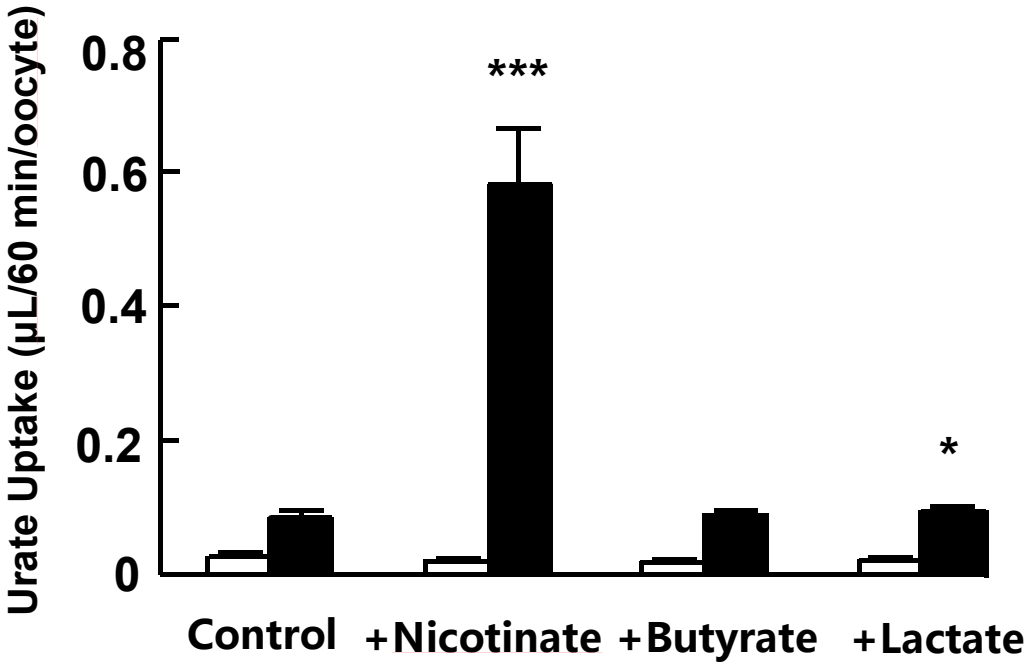


Fig. 2-3 Effect of different monocarboxylates on urate uptake by SMCT1-URAT1 co-expressing oocytes. SMCT1-URAT1 co-expressing oocytes (closed bar) and water-injected oocyte (open bar) were preincubated in ND96 buffer (pH7.4 containing 1.5 mM sodium nicotinate, sodium butyrate, or sodium lactate at 25^{o}C for 60 min before urate uptake. Then, the oocytes were washed three times with ND96 buffer (pH7.4) and were transferred to a 24-well plate containing ND96 buffer (pH7.4) and 10 μM [^{14}C]urate for the uptake study. Each point represents the mean ± SEM from 8-9 oocytes. Student's t-test: ***p<0.001, *p<0.05, compared with control.

2-3-3 Accumulation of nicotinate by *Xenopus* oocytes co-expressing SMCT1 and URAT1

Fig. 2-4 depicts the accumulation of nicotinate in oocytes co-expressing SMCT1-URAT1 and oocytes expressing URAT1 alone. When the uptake time was set at 60 min, accumulation of nicotinate in SMCT1-URAT1 co-expressing oocytes exhibited a concentration-dependence and saturated at 1 mM (Km: 241 ± 36 μM) (**Fig. 2-4A**). When nicotinate concentration was set at 1 mM, accumulation of nicotinate in SMCT1-URAT1 co-expressing oocytes reached steady-state at 60 min (**Fig. 2-4B**). In oocytes expressing URAT1 alone, the accumulation of nicotinate is quite small compared with that of SMCT1-URAT1 co-expressing oocytes and was comparable with that in water-injected control oocytes.

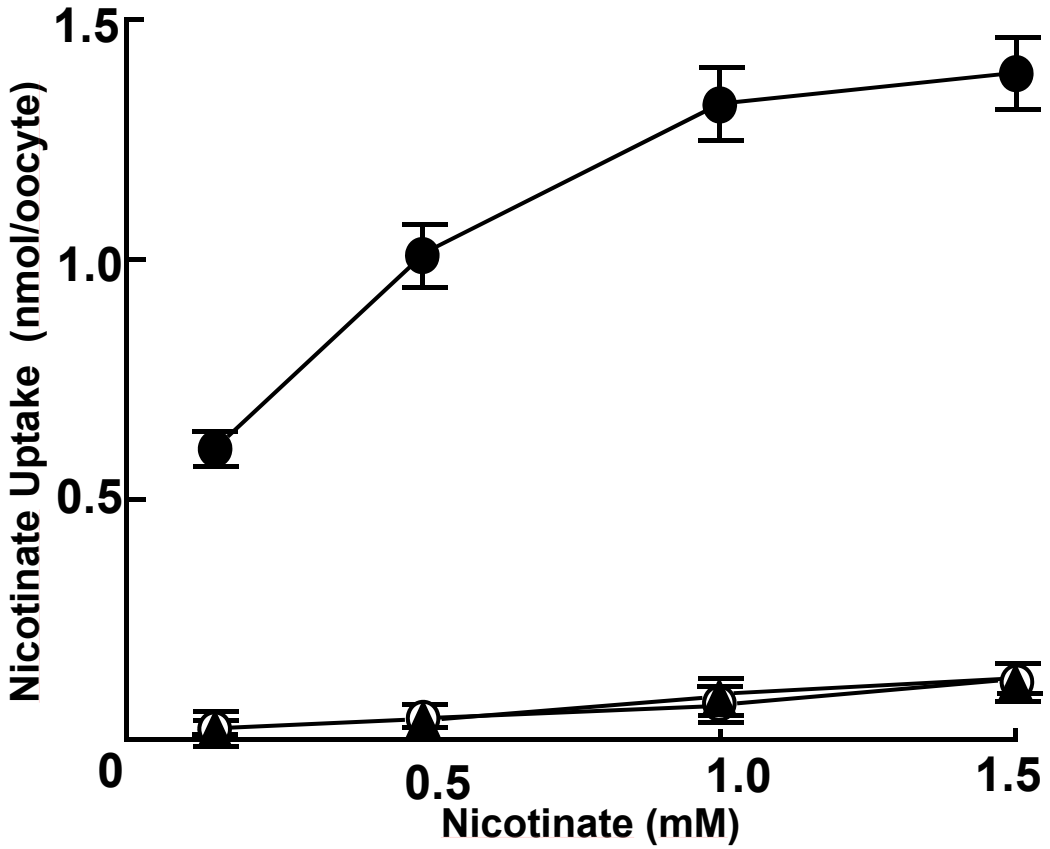
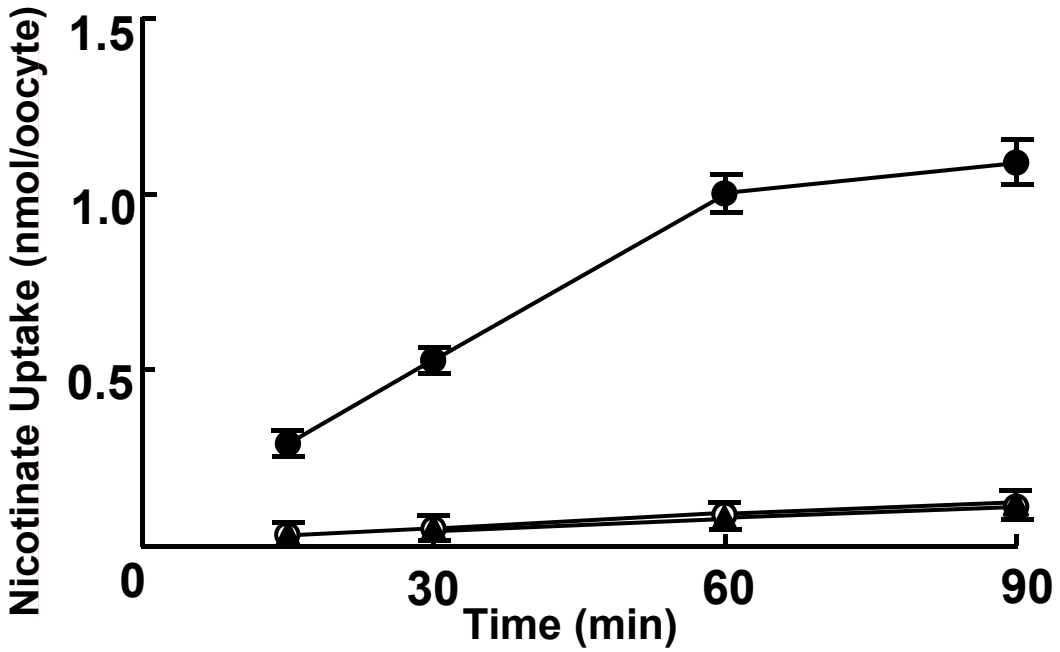


Fig. 2-4 Accumulation of nicotinate in oocytes expressing SMCT1 and URAT1. A. Uptake of nicotinate was investigated in SMCT1-URAT1 co-expressing oocytes (closed circle), oocytes expressing URAT1 alone (open circle), or water-injected oocytes (closed triangle) with nicotinate concentration ranged

from 0.15 to 1.5 mM. The uptake time was set at 60 min. Each point represents the mean ± SEM from 9-10 oocytes.



B. Uptake of nicotinate was investigated in SMCT1-URAT1 co-expressing oocytes (closed circle), oocytes expressing URAT1 alone (open circle), or water-injected oocytes (closed triangle) with uptake time ranged from 15 to 90 min. Nicotinate concentration was set at 1 mM. Each point represents the mean ± SEM from 9-10 oocytes.

2-3-4 Influence of preincubation concentration of nicotinate on its stimulation effect of URAT1-mediated urate uptake

Influence of nicotinate concentration on the stimulation effect was studied in SMCT1-URAT1 co-expressing oocytes after preloading 0, 0.15, 0.5, and 1 mM nicotinate for 60 min. Urate uptake was stimulated by nicotinate in SMCT1-URAT1 co-expressing oocytes in a concentration-dependent manner (**Fig. 2-5A**). Effect of preloading of nicotinate on urate uptake by oocytes expressing URAT1 alone was also studied. An increase of urate uptake by URAT1 expressing oocytes was also observed after preloading nicotinate compared without preloading of nicotinate. Accordingly, co-expression of URAT1 and SMCT1 is effective to activate URAT1-mediated uptake of urate.

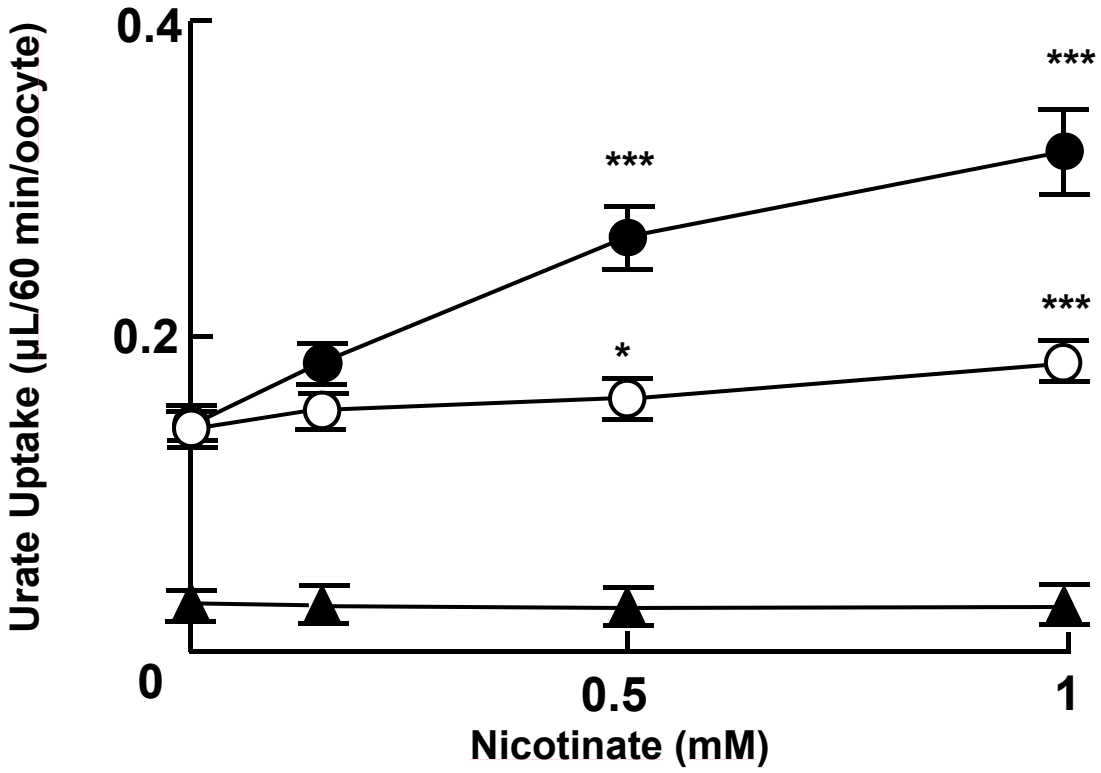


Fig. 2-5 Influence of preincubation time and concentration of nicotinate on the stimulation of urate uptake by SMCT1-URAT1 co-expressing oocytes.

A. SMCT1-URAT1 co-expressing oocytes (closed circle), oocytes expressing URAT1 alone (open circle), or water-injected oocytes (closed triangle) were preincubated in ND96 buffer (pH7.4) containing 0, 0.15, 0.5, and 1mM sodium nicotinate at 25^{o}C for 60 min before urate uptake. Uptake of urate was carried out in ND96 buffer (pH7.4) containing 10 µM [^{14}C]urate at 25^{o}C for 60 min. Each point represents the mean ± SEM from 7-9 oocytes. Student's t-test: ***p<0.001, *p<0.05, *vs.* control (0 min preincubation).

2-3-5 Influence of preincubation time of nicotinate on the stimulation effect of URAT1-mediated urate uptake

The influence of nicotinate preincubation time on stimulatory effect was studied after preincubation with 1 mM nicotinate for 0, 15, 30, 60 min before initiation of urate uptake. With an increasing preincubation time of nicotinate, higher stimulation of urate uptake was observed in SMCT1-URAT1 co-expressing oocytes for up to 30 min as shown in **Fig. 2-5B**.

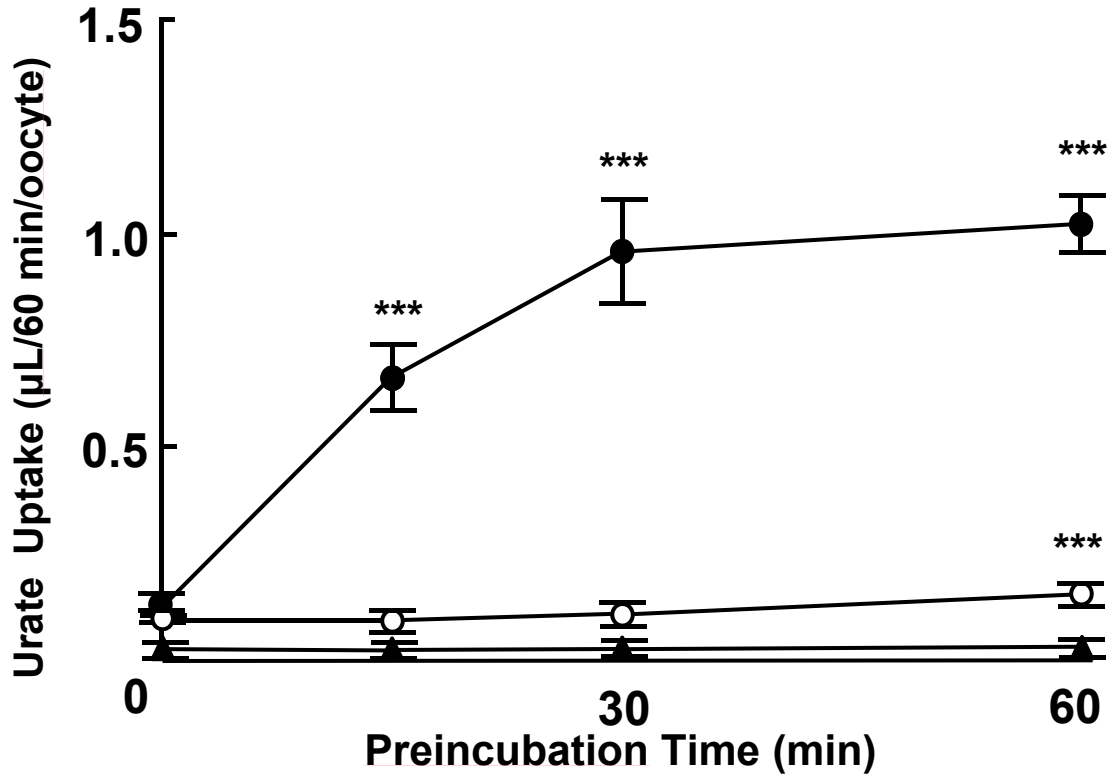


Fig. 2-5B SMCT1-URAT1 co-expressing oocytes (closed circle), oocytes expressing URAT1 alone (open circle), or water-injected oocytes (closed triangle) were preincubated in ND96 buffer (pH7.4) containing 1 mM sodium nicotinate for 0, 15, 30, 60 min before urate uptake. Then, the oocytes were washed three times with ND96 buffer (pH7.4) and were transferred to a 24-well plate containing ND96 buffer (pH7.4) and 10 μM [^{14}C]urate for the uptake study. Each point represents the mean ± SEM from 8-9 oocytes. Student's t-test: ***p<0.001, *p<0.05, *vs.* control (0 min preincubation).

2-3-6 Influence of sodium ions on the stimulation effect

To verify the mechanism of SMCT1-mediated stimulation effect, the influence of sodium ions and SMCT1 inhibitors was investigated. SMCT1-URAT1 co-expressing oocytes and oocytes expressing URAT1 alone were preincubated in ND96 buffer with 1 mM nicotinate for 60 min in the presence or absence of sodium ions by replacing with *N*-methylglucamine. For the uptake of urate, oocytes were transferred to uptake medium with or without sodium ions. **Fig. 2-6** shows that uptake of urate by SMCT1-URAT1 co-expressing oocytes was drastically decreased when removing sodium ions in the preloading condition. Meanwhile, the uptake of urate by oocytes expressing URAT1 alone was unchanged in the presence or absence of sodium ions. The apparent sodium ion dependence in preincubation medium is ascribed to the sodium dependent uptake of nicotinate by SMCT1. Slight, but not significant, effect of sodium ions during uptake of urate may be explained by the re-uptake of nicotinate by URAT1, which was effluxed by exchange with urate via URAT1.

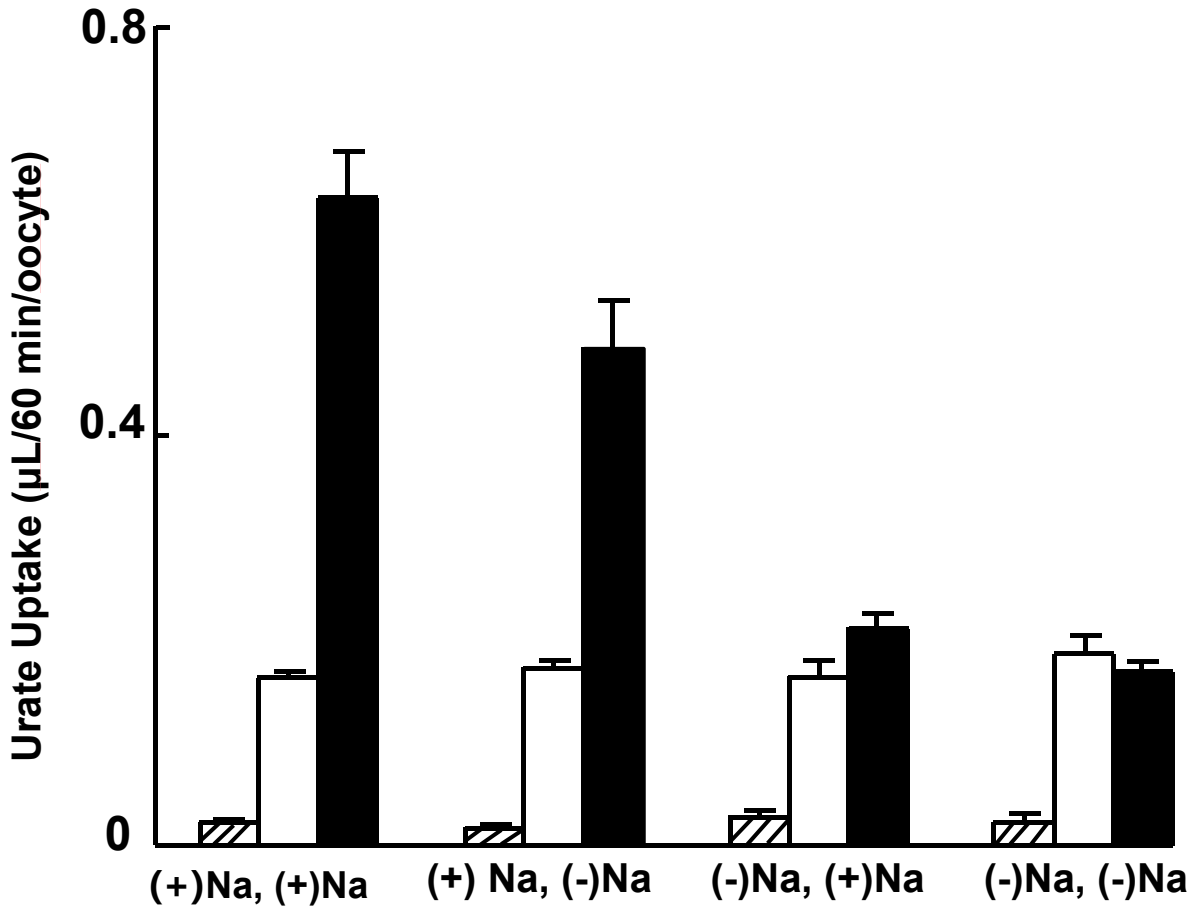


Fig. 2-6. Influence of sodium ions on the stimulation of urate uptake by SMCT1-URAT1 co-expressing oocytes.

SMCT1-URAT1 co-expressing oocytes (closed bar), oocytes expressing URAT1 alone (open bar) and water-injected oocytes (slashed bar) were preincubated in ND96 buffer (pH7.4) containing 1 mM nicotinate in the presence or absence of sodium ions. Then, the oocytes were washed three times with ND96 buffer or sodium free ND96 buffer (pH7.4), and were transferred to a 24-well plate containing ND96 buffer (pH7.4) and 10 µM [^{14}C]urate for the uptake study in the presence or absence of sodium ions. Each point represents the mean ± SEM from 8-11 oocytes.

2-3-7 Influence of SMCT1 inhibitors on the stimulation effect

When SMCT1-URAT1 co-expressing oocytes were preincubated with 5 mM butyrate or propionate, uptake of nicotinate by SMCT1-URAT1 co-expressing oocytes had 85% and 76% decrease, respectively (**Fig. 2-7A**). When oocytes expressing both URAT1 and SMCT1 or URAT1 alone were preincubated with 5 mM butyrate or propionate, the stimulatory effect was also significantly decreased by 78% and 73%, respectively, as shown in **Fig. 2-7B**. Nicotinate uptake was comparable with that in oocytes expressing URAT1 alone, while the uptake was still much higher than that by water-injected control oocytes. Thus, these compounds are suggested to be inhibitors but not exchanged well with urate via URAT1 as shown in **Fig. 2-3** (butyrate). Accordingly, observed decrease in urate uptake in the presence of butyrate and propionate may be due to the decreased supply of nicotinate into oocytes by inhibiting nicotinate uptake by SMCT1, resulting in the decreased stimulation effect of urate via URAT1.

A:

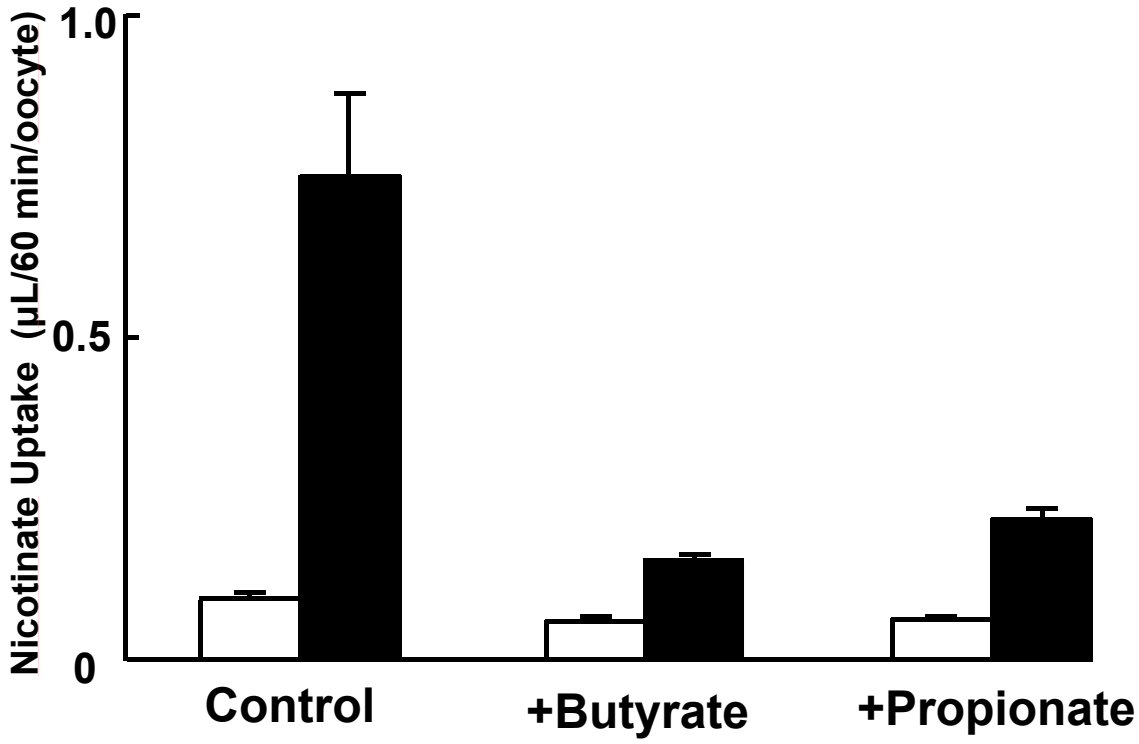
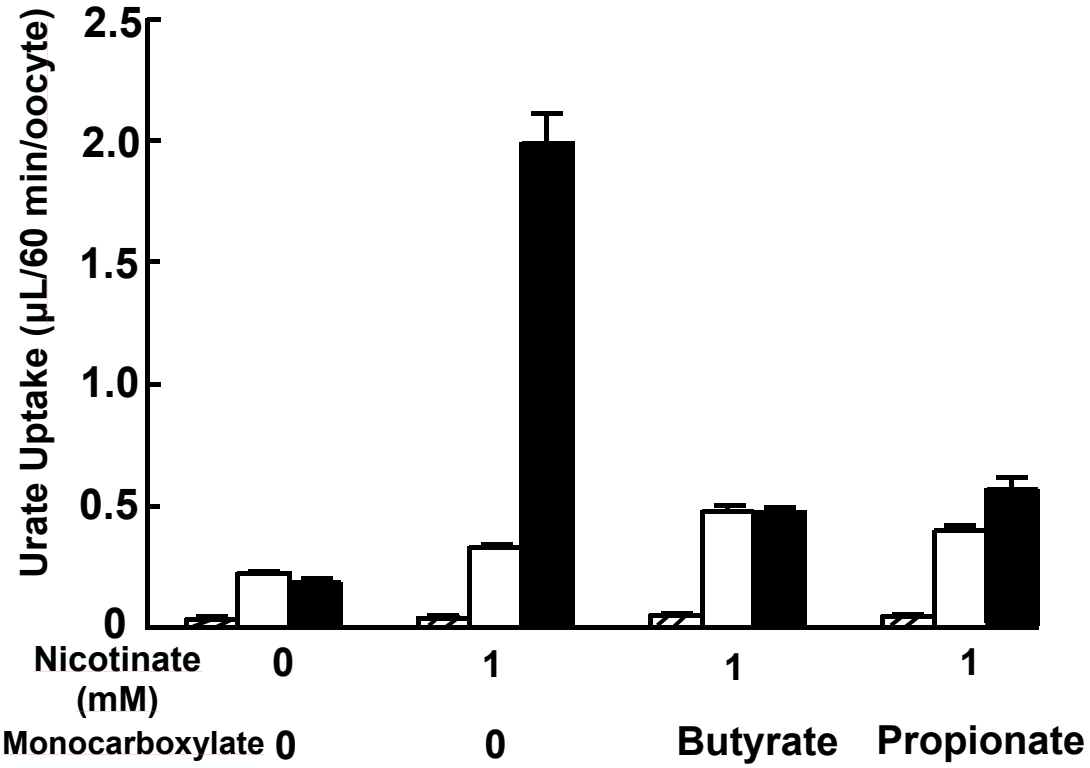


Fig. 2-7 Influence of SMCT1 inhibitors on the uptake of nicotinate and stimulation effect on urate uptake by SMCT1-URAT1 co-expressing oocytes.
A. Influence of butyrate and propionate on nicotinate uptake by SMCT1-URAT1 co-expressing oocytes (closed bar) was investigated. Nicotinate uptake was carried out for 60 min with 1 mM nicotinate in the presence or absence of 5 mM of butyrate or propionate. Open bar: uptake of nicotinate by water-injected oocytes. Each point represents the mean ± SEM from 10 oocytes.

B:



B. SMCT1-URAT1 co-expressing oocytes (closed bar), oocytes expressing URAT1 alone (open bar), or water-injected oocytes (slashed bar) were preincubated in ND96 buffer (pH7.4) with or without 1 mM nicotinate in the presence or absence of 5 mM of butyrate or propionate for 60 min before urateuptake. Then urate uptake was carried out for 60 min. Each point represents the mean ± SEM from 10 oocytes.

2-4 Discussion

In this study, we investigated the functional cooperation between human URAT1 and SMCTs in the reabsorptive transport of urate *in vitro*. Because SMCT1 and SMCT2 have similar function in the transport of monocarboxylates and are expressed at the tubular cells, one of them (SMCT1) was chosen to prove the cooperation of SMCT1 and URAT1 for urate reabsorption. Initially, a SMCT1-URAT1 double expressing system was established in *Xenopus* oocytes to express both SMCT1 and URAT1. A *trans*-stimulation effect of L-lactate was observed when comparing the uptake of urate by oocytes expressing URAT1 alone and by SMCT1-URAT1 co-expressing oocytes after preincubating the oocytes with L-lactate. Considering the possible differences in the expression level of URAT1 between URAT1-alone and SMCT1-URAT1 co-expressing oocytes, which may also explain apparent difference of urate uptake between these two types of oocytes, the effect of nicotinate, butyrate and L-lactate on urate uptake was studied in SMCT1-URAT1 co-expressing oocytes to find a better counter monocarboxylate to be exchanged with urate via URAT1. Since nicotinate showed the highest stimulation effect, we further studied the stimulation effect by changing the preincubation time and concentration of nicotinate. Finally, to confirm that URAT1 activity is stimulated by SMCT1 function, the influence of removing sodium ions from uptake buffer and adding SMCT1 inhibitors were investigated. The results of this study clearly demonstrated the functional cooperation of SMCT1 in urate reabsorption via URAT1.

Affinities of nicotinate, L-lactate, and butyrate on SMCT1 have been reported in the previous studies. K_m values for nicotinate, L-lactate and butyrate are 230 μM, 81 μM, and 235 μM, respectively [81, 89]. Because they have a K_m value around or lower than 250 μM, 1.5 mM was initially selected as the preloading concentration to provide enough monocarboxylate for the exchange of urate via URAT1. In **Fig. 2-3**, while SMCT1-URAT1 co-expressing oocytes were

preincubated with monocarboxylates, nicotinate showed higher stimulation effect. This phenomenon may be explained by the different affinity of nicotinate and lactate in the exchange with urate via URAT1 and is consistent with the results of a previous study in which a different stimulation effect was observed with direct injection of them into oocytes expressing URAT1 alone [54]. Physiologically, normal serum lactate concentration is around 1.5 mM, which is considered to provide the major driving force for URAT1. Although lactate concentration used in this experiment was close to normal serum lactate concentration, lactate showed much smaller stimulation effect than nicotinate under current experimental condition. The discrepancy between *in vivo* and *in vitro* might be explained by the reason that any monocarboxylates other than lactate and nicotinate may also be involved in the trans-stimulation of URAT1 *in vivo*. Also, intracellular lactate concentration may be different between this experimental model and *in vivo* renal proximal tubular cell. Renal cell might show lower lactate concentration, so URAT1-mediated urate uptake is more sensitive to stimulation by SMCT1-mediated lactate transport in the renal cells *in vivo*. Although physiological relevance such as serum monocarboxylate concentration and stimulation of URAT1 may not be clear at present, it is clear that SMCT substrates increase the apparent urate uptake activity by URAT1, demonstrating functional cooperation of these two transporters.

The accumulation of nicotinate showed concentration- and time-dependence and attained maximum accumulation of nicotinate at 1 mM and 60 min (**Fig. 2-5A**). In accordance with such accumulation of nicotinate, urate accumulation in oocytes was increased with an increase of concentration and preincubation time of nicotinate (**Fig. 2-5**). These results indicated that a preincubation of nicotinate at 1 mM for 60 min is sufficient in the following experiments, while higher concentration (>1 mM) or longer preloading time (>60 min) might increase the nicotinate taken up by oocytes. When preincubating oocytes expressing URAT1 alone in ND96 buffer containing nicotinate, an increase of

urate uptake by oocytes expressing URAT1 alone was observed (**Fig. 2-5A**). This can be explained by the diffusion and/or carrier-mediated uptake of nicotinate from the uptake medium into oocytes by endogenous transporter which can be exchanged with urate via URAT1.

As SMCT1 is a sodium dependent transporter [81, 89] but URAT is sodium independent, function of SMCT1 in SMCT1-URAT1 co-expressing oocytes should be depressed by removing sodium ions from the uptake buffer, thus reducing the nicotinate taken up by SMCT1-URAT1 co-expressing oocytes in exchange with urate and affecting the observed stimulation effect. Because the uptake of urate via URAT1 is not affected by sodium ions [54], removing sodium ions from uptake buffer should not affect the function of URAT1 in SMCT1-URAT1 co-expressing oocytes. As expected, by removing the sodium ions during the preloading of nicotinate, uptake of urate in SMCT1-URAT1 co-expressing oocytes was greatly reduced (**Fig. 2-6**). The addition of butyrate and propionate also led to the decrease of the stimulation effect (**Fig. 2-7B**), because butyrate and propionate are inhibitors of SMCT1 and can reduce the accumulation of nicotinate in SMCT1-URAT1 co-expressing oocytes (**Fig. 2-7A**). These results clearly indicate that the increase of urate uptake in SMCT1-URAT1 co-expressing oocytes was due to the exchange of nicotinate taken up by SMCT1 with urate and demonstrated the cooperative relationship between SMCT1 and URAT1 in urate reabsorption.

It has been suggested that URAT1 and SMCTs can be physically linked through PDZK1, resulting in a possible functional relationship [90-92]. Both of SMCT1 and SMCT2 are known to be binding partners of PDZK1 [84]. Accordingly, the present study provides the functional cooperation of SMCTs and URAT1 in the reabsorptive transport of urate via URAT1. Expressed in colon, ileum, kidney and thyroid gland [93], SMCTs was recognized as a tumor

suppressor in the previous studies [94-99]. This study reveals the pivotal of SMCTs in modulation of renal urate transport.

In conclusion, this is the first study providing direct evidence for the hypothesis that SMCTs could enhance URAT1-mediated urate uptake. Results from this study demonstrate the cooperative relationship of URAT1 and SMCTs and indicate that SMCTs may be used as a potential target for the alteration of renal handling of urate indirectly. Also, we should be careful in considering the serum uric acid level by clinically used drugs or other factors including diseases since change in activity of SMCTs affect reabsorptive activity of urate.

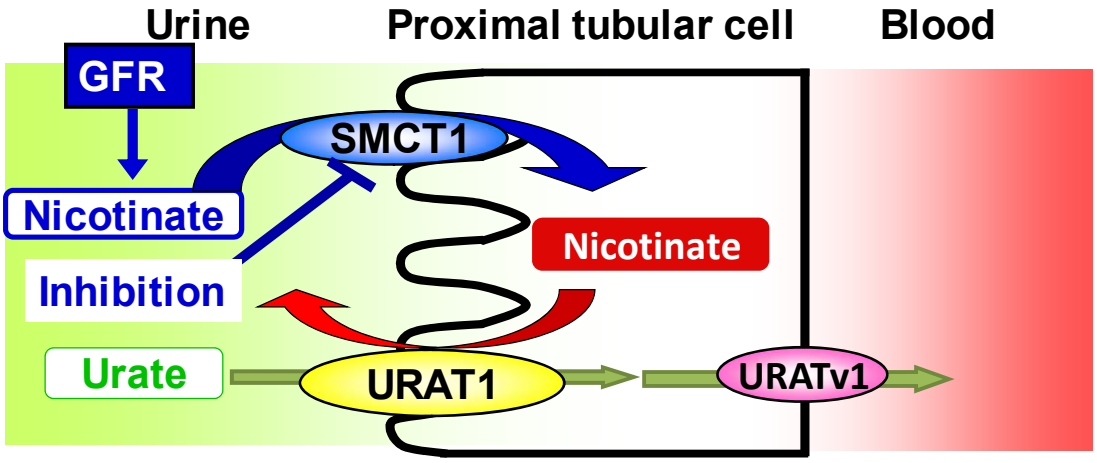


Fig. 2-8 Indirect regulation of serum urate level by affecting SMCT1.

Chapter 3 A putative mechanism of lowered serum urate level by whisky

Abstract:

Clinical studies show that moderate consumption of whisky results in an increase of renal excretion of urate into urine and a decrease of the serum urate level. The effects of whisky congeners on urate transporters responsible for the reasorptive transport of urate were examined using *Xenopus* oocytes gene-expressing system. Urate uptake by *Xenopus* oocytes expressing human URAT1 or human URATv1 was investigated in the presence or absence of congeners. Congeners from 12-year and 18-year whiskies showed an inhibitory effect of the urate uptake by URAT1 with an IC_{50} value of 0.084 ± 0.011 and 0.042 ± 0.0056 mg/mL, respectively. For urate uptake by URATv1, congeners from 12-year whisky exhibited an inhibitory effect of about 24.4 ± 3.0% inhibition only at 1 mg/mL. Similarly, congeners from 18-year whisky showed 22.5 ± 1.6% inhibition only at 1 mg/mL. There was no significant difference between the inhibitory effect of congeners from 12 years on URATv1-mediated urate uptake and that by congeners from whisky stored for 18 years. At lower concentrations, there was no inhibitory effect observed for both of the congeners. Results of this study suggested that decreased serum urate level after whisky consumption may be due to the inhibition of URAT1 by congeners.

3-1 Introduction

Lifestyle and dietary factors are closely related to human health. Daily exposure of food is inevitable for all human beings. Influence of different kinds of food and beverage on human health has been realized since ancient times. At present, food therapy has been regarded as one of the most important approaches of Traditional Chinese Medicine for the cure or prevention of a number of diseases [100]. With the development of modern science, an increasing number of scientific evidences and reports have emerged in recent years illustrating the mechanisms of the impact of food and beverage on human health and providing new evidences at the same time.

Urate is the final product of purine metabolism in humans. Because humans lack urate oxidase, urate cannot undergo further oxidation reaction to form allantoin, a more soluble compound. Serum urate level is maintained by the generation and excretion of urate. Purine intake from food is an important source of serum urate accounting for approximately one third of daily urate load [101]. Intake of purine-rich food has been associated with incident of hyperuricemia and gout attack [102-103]. Acute intake of purine-rich food can raise the risk of recurrent gout attack as high as five fold in patients suffering from gout [104]. It is also found that impact of purine from animal sources on recurrent gout attack was higher than that from plant sources. Thus, intake of low purine food and reduced consumption of animal-source food have been suggested in the prevention and treatment of hyperuricemia and gout [105-107].

Serum urate level is related to alcohol and beverage intake as well. Habitual intake of alcoholic beverages and hyperuricemia has been associated in many reports. In a recent study carried out in Japanese men, alcohol intake and risk of the incident hyperuricemia are correlated at a dose dependent manner.

Alcohol intake is responsible for 21.6% of hyperuricemia occurrence in Japanese men [108]. Alcohol consumption can also increase the risk for gout attack in men [109]. For the relationship between alcohol consumption and high urate level, two putative mechanisms have been proposed. One is that alcohol intake can increase lactate level during alcohol oxidation and lactate is an important substrate of sodium-coupled monocarboxylate transporters (SMCTs) for the exchange of urate via urate transporter 1 (URAT1). SMCTs have functional cooperative relationship with URAT1 as is demonstrated in Chapter 2 and has been reported in our previous study [110]. Increased lactate may enhance the reabsorptive transport of urate through URAT1. The other might be due to the increased adenosine triphosphate (ATP) degradation to adenosine monophosphate (AMP) during alcohol metabolism [111-113]. Because urate is synthesized from adenosine, the production of urate will be enhanced after alcohol intake.

Consumption of alcoholic beverages has been a feature of many cultures throughout the world since ancient times, and especially in some Asian countries, is often associated with social gatherings. Many studies have shown that regular alcohol consumption is a risk factor for hyperuricemia and gout attack, though moderate consumption of some kinds of alcoholic beverages may provide health benefits. For example, moderate red wine drinking may help to reduce the risk of coronary heart disease [114]. Such effect might be attributed to the nonvolatile substances (called congeners) generated during brewing maturation processes. It has been reported that congeners contained in whisky exhibit diverse biological activities including protection of the gastrointestinal tracts, inhibition of melanogenesis and suppression of NO production [115-118]. Recent clinical studies show that consumption of whiskey results in an increase of renal excretion of urate as well as a decrease of the serum urate level (SUA) [119], although the mechanism remains to be determined. Therefore, understanding effect of the congeners on alteration in

urate reabsorption may help us to find the molecular details of decreased SUA after whisky consumption.

In order to delineate the mechanism by which serum urate level is reduced after whisky consumption presumably by certain congeners, it is essential to understand how whisky congener is involved in alteration in serum urate level. Urate is poorly hydrophilic to permeate the membranes of proximal tubular cells, thus membrane transporters play a pivotal role in its reabsorptive and secretory transcelluar transport and maintain SUA [120]. Till now, a number of membrane transporters involved in urate transport have been identified including organic anion transporter family members (OATs) and breast cancer resistance protein (BCRP) [54]. Among these transporters, urate transporter 1 (URAT1/*SLC22A12*), which is a member of OATs, is localized on the apical side of renal proximal tubular cells and has been characterized as a transporter mainly responsible for renal reabsorption of urate from lumen into blood [54]. Since kidney handles approximately 70% of urate in the body, renal urate transporters are of great significance. Currently, URAT1 has already been a target for the development of novel anti-hyperuricemia compounds. On the basolateral side of renal proximal tubule, a voltage-driven urate transporter (URATv1/ *SLC2A9*) functions as a urate transporter mediating the urate transport from proximal tubular cell into blood [64]. Cooperative role of URAT1 and URATv1 in renal reabsorption of urate has been demonstrated by the previous study conducted in our laboratory [121]. In order to explain the phenomenon of decreased SUA after drinking whisky, in the present study we examined whether whisky congeners have any inhibitory effect on URAT1 and URATv1 mediated reabsorptive transport of urate (putative mechanism is shown in **Fig.3-1**).

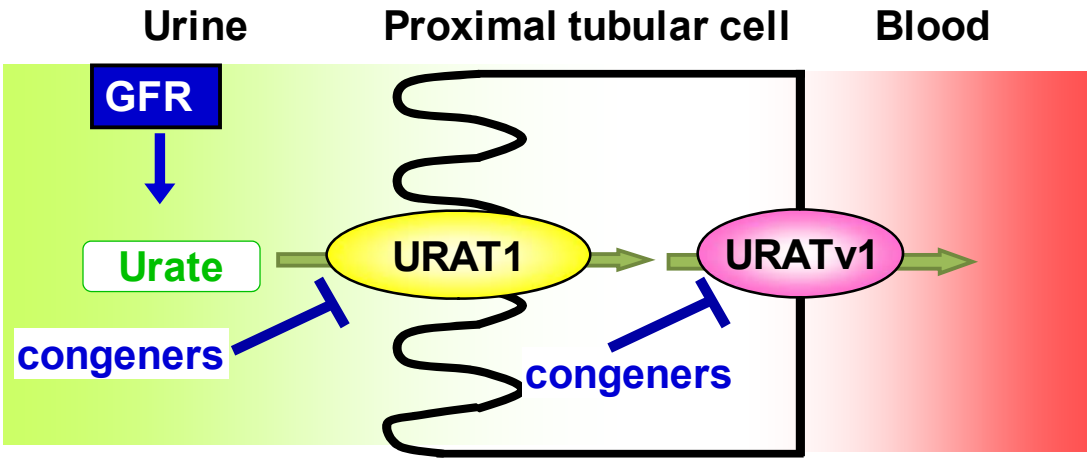


Fig. 3-1 Hypothesized model of congener's effect on renal urate reabsorptive transport. Whisky congeners inhibit URAT1and URATv1-mediated urate reabsorptive transport, reducing the serum urate level.

3-2 Materials and Methods

3-2-1 Materials

[^{14}C]Urate (1.96 TBq/mol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Congeners from 12 and 18-year old whiskies were supplied by Suntory Holdings (Osaka, Japan). Clearsol-I was the product of Nacalai Tesque (Kyoto, Japan). All other reagents were of analytical grade.

3-2-2 Preparation of URAT1 and URATv1 cRNA

Human URAT1 and URATv1 cRNA were *in vitro* transcribed and *Xenopus* oocytes expressing these transporters are prepared as previously described in Chapter 2-2. The whole study was approved by the Institutional Animal Care and Use Committee of Kanazawa University.

3-2-3 Uptake study by *Xenopus laevis* oocyte

As for uptake of urate by oocytes expressing URAT1 or URATv1, oocytes were treated as previously described in Chapter 2-2. Uptake of [^{14}C]urate (20 μM) by oocytes expressing URAT1 was carried out for 60 min at 25 ^{o}C in pH7.4 ND 96 buffer free of Cl^- ions. [^{14}C]Urate uptake by oocytes expressing URATv1 was performed for 60 min in ND96 buffer not containing Na^+ ions. At the end of uptake experiment, oocytes were washed three times with ice-cold uptake buffer, and each oocyte was lysed in 5% sodium dodecyl sulfate solution (50 μL) to quantify radioactivity on a liquid scintillation counter (LSC-5100, Aloka, Tokyo).

pH7.4 ND96 buffer free of Cl^- ions:
96 mM sodium gluconate
2 mM potassium gluconate
1 mM magnesium gluconate
1.8 mM calcium gluconate

5 mM HEPES

Adjust pH to 7.4.

ND96 buffer not containing Na^+ ions

98 mM KCl

1 mM $MgCl_2$

1.8 mM $CaCl_2$

5 mM HEPES

Adjust pH to 7.4.

3-2-4 Data Analysis

Inhibitory effect of congeners on urate uptake was calculated by KaleidaGraph 4.0 (Synergy Software, Reading, PA) with following equation:

% of control = 100 × $IC_{50}/(IC_{50} + I)$,

in which IC_{50} is the 50% inhibitory concentration;

I is the concentration of the congeners used in the experiment.

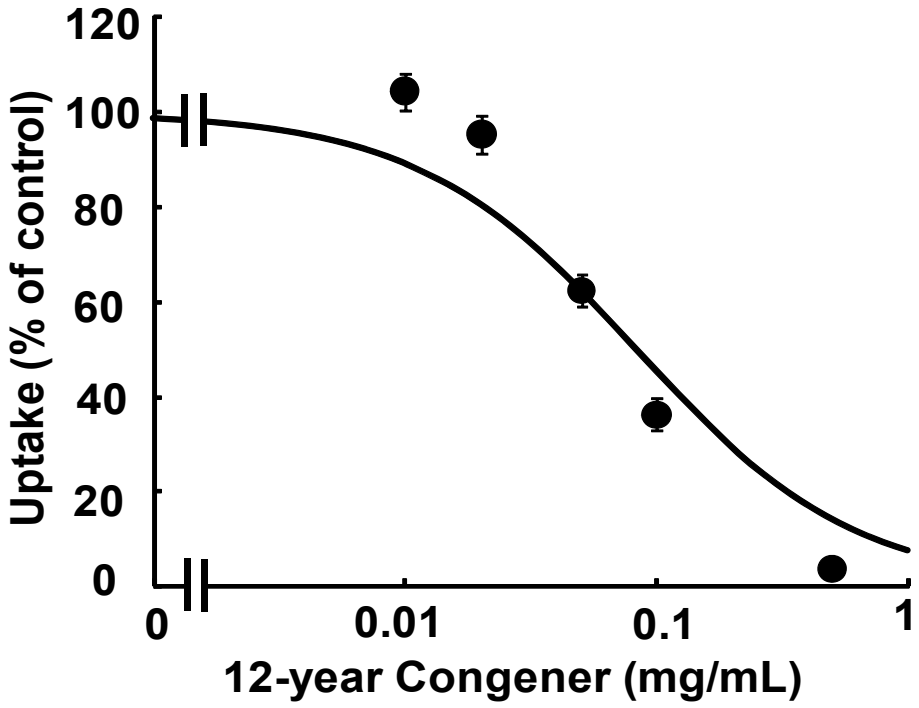
All data were expressed as mean ± SEM. Statistical significance was analyzed by Student's t-test. A *p* value less than 0.05 was considered statistically significant.

3-3 Results

3-3-1 Effect of whisky congeners on URAT1 mediated urate uptake

Influence of whisky congeners on URAT1- and URATv1-mediated urate uptake was studied to delineate their potentials to lower SUA after whisky consumption. Congeners from 12 and 18–year old whiskies were tested for their effect on URAT1-mediated [^{14}C]urate uptake at a concentration range from 0.01 to 0.5 mg/mL, and 0.002 mg/mL to 0.5 mg/mL, respectively. Both congeners inhibited URAT1-mediated urate uptake in a concentration-dependent manner (Fig. 3-2 A and B). IC_{50} values for congeners from 12 and 18–year old whiskies were estimated at 0.084 ± 0.011 (Fig. 3-2A) and 0.042 ± 0.0056 mg/mL (Fig. 3-2B), respectively.

A:



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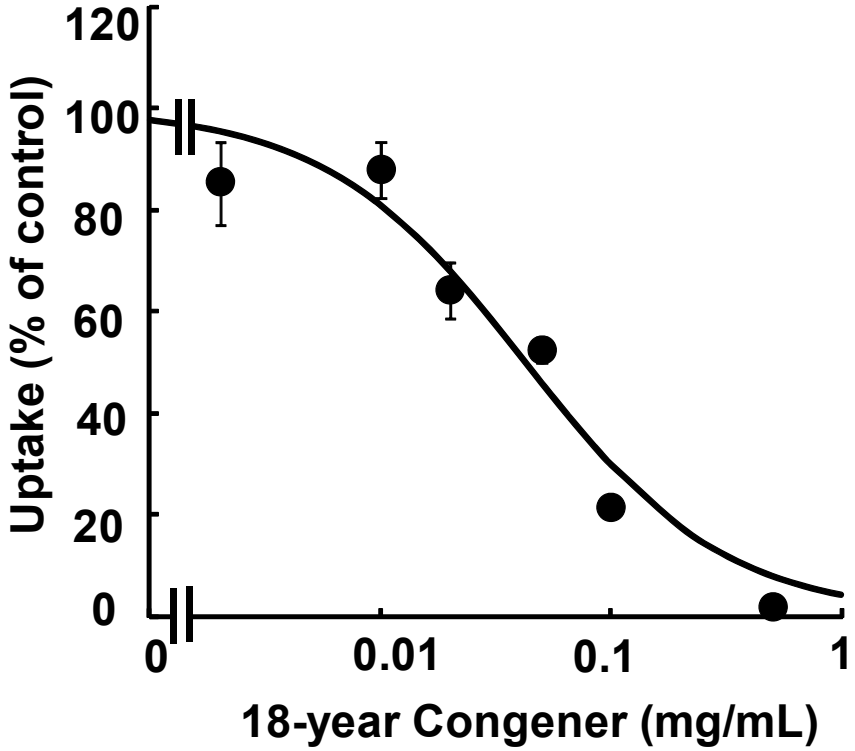
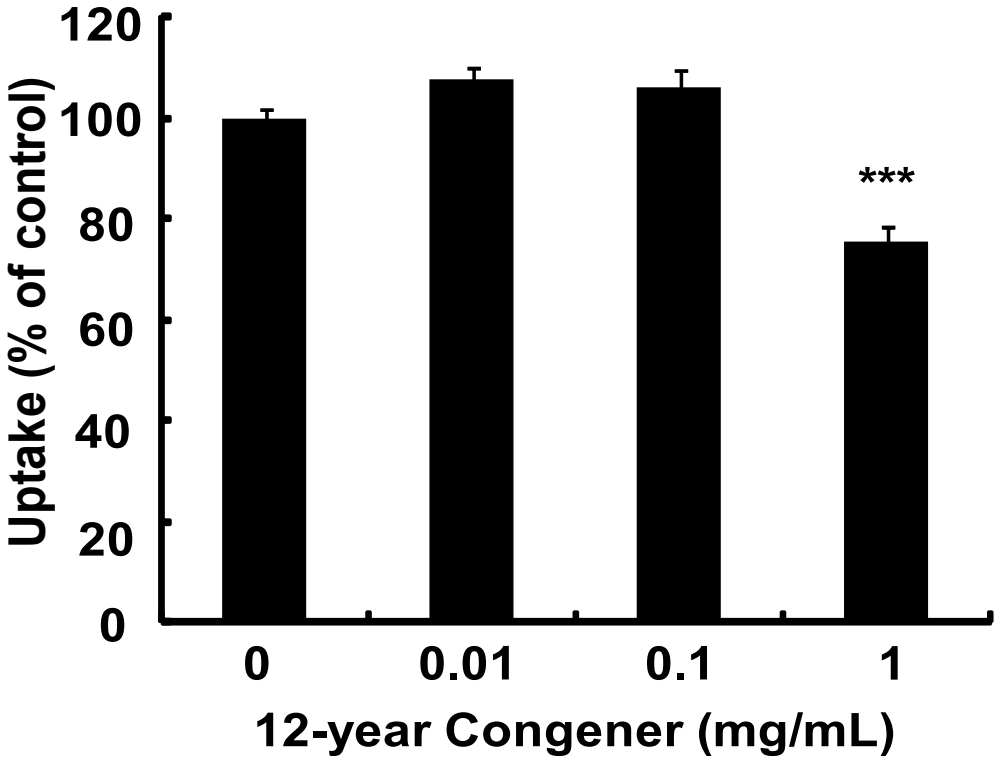


Fig. 3-2 Inhibitory effect of 12 (A) and 18-year (B) old whisky congeners on URAT1-mediated urate uptake. Each point represents the mean ± SEM from 10 oocytes.

3-3-2 Effect of whisky congeners on URATv1 mediated urate uptake

To determine whether congeners from 12 and 18-year old whiskies have any inhibitory effect on URATv1-mediated urate uptake, the congeners were tested for their inhibitory effects on uptake of urate by oocytes expressing URATv1. **Figure 3-3A** shows that congeners from 12-year old whisky reduced the uptake by 24.4 ± 3.0% at 1mg/mL. Similarly, congeners from 18-year old whisky showed 22.5 ± 1.6% inhibition at 1mg/mL (**Fig. 3-3B**). No significant difference was found between the effects of the congeners from 12 and 18-year old whiskies at 1 mg/mL. At lower concentrations (0.1 mg/mL and 0.01 mg/mL), both congeners showed no inhibitory effect on URATv1-mediated urate uptake (**Fig. 3-3A** and **Fig. 3-3B**).

A:



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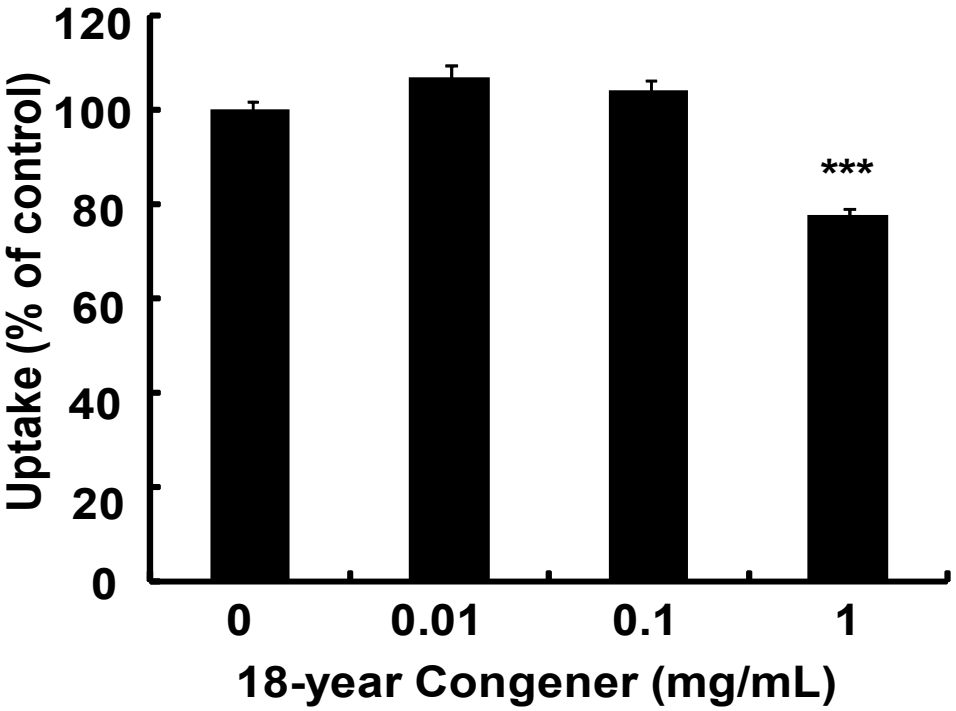


Figure 3-3 Inhibitory effect of 12 (A) and 18-year (B) old whisky congeners on URATv1-mediated urate uptake. Each point represents the mean ± SEM from 10 oocytes. Student's t-test: ***, p<0.001 compared with control.

3-4 Discussion

In the present study, we aimed at investigating the possible mechanism of lowered serum urate level after whisky consumption. Due to the important role of URAT1 and URATv1 in regulating serum urate level, we investigated the effect of whisky congeners on these urate transporters. Both 12-year and 18-year congeners showed inhibitory effect on URAT1 mediated urate uptake (**Fig. 3-2**). Interestingly, 18-year old whisky congener exhibited stronger inhibitory effect on URAT1-mediated urate uptake compared with that by 12-year old one. Spirit maturation is an important process responsible for the quality, flavor, color and taste of whisky. During the long maturation time, many substances are generated including aromatic aldehydes, phenols and acids [122-123], either by migration of oak constituents or by maturation of spirit. It has been reported that 18-year congener has higher contents of polyphenolic compounds compared with 12-year congener, such as gallic acid, ellagic acid and lyoniresinol [124]. Because content of congeners in whisky is associated with maturation time [123], longer maturation of whisky in oak casks might generate more substances, resulting in more enriched congeners, where higher yield of active ingredients may be responsible for greater inhibition of URAT1. This might explain observed stronger effect of congener from18-year old whisky.

Congeners showed inhibitory effect on URATv1-mediated urate uptake at 1 mg/mL (**Fig. 3-3**). At lower concentration, congeners seem to have no inhibitory effect on URATv1-mediate urate uptake. Because 1 mg/mL is close to the original concentration of congeners in whisky, these congeners are unlikely to reach such high concentration *in vivo*. Therefore, the inhibition of URATv1 mediated urate uptake by whisky congeners is not likely to exist after whisky consumption.

In conclusion, the current study shows that congeners from whiskies matured for 12 and 18 years can inhibit URAT1 and URATv1 mediated urate uptake under experimental conditions employed in the present study. Considering the concentration of congeners *in vivo*, inhibition of URAT1 by congeners more likely contributes to reduce SUA after whisky consumption (**Fig. 3-4**).

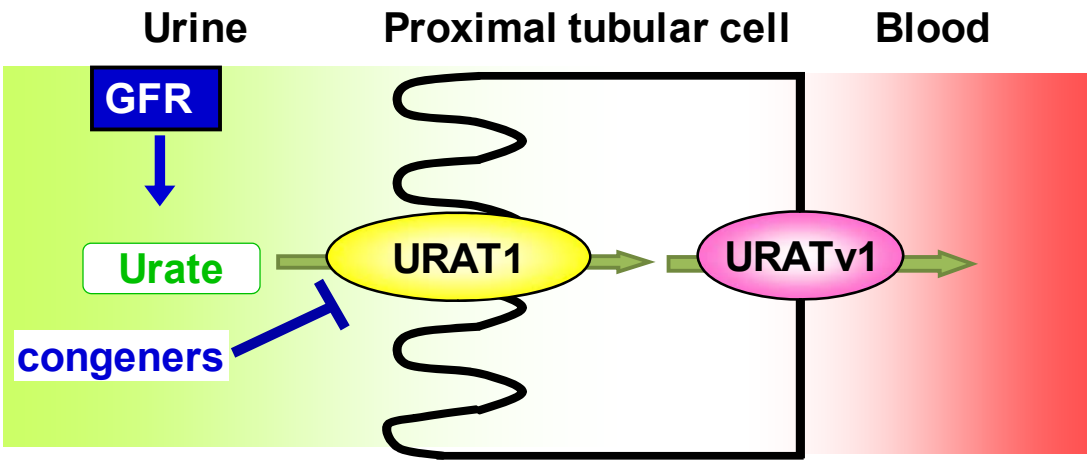


Fig. 3-4 Proposed mechanism for lowered serum urate level after whisky consumption. Whisky congeners may reduce serum urate level by inhibition of URAT1-mediated urate reabsorptive transport.

Chapter 4 Indoxyl sulfate upregulates BCRP expression in intestinal cell line

Abstract

In the state of chronic renal disease (CKD), renal function is greatly reduced. However, the rise of serum urate level is not much, compared with other solutes. Compensatory increased non-renal urate excretion might be contributing to explain this phenomenon. Intestinal BCRP is significantly involved in controlling serum uric acid level and previous study shows that Bcrp expression is increased in CKD rats, while it is not known whether such an increase of Bcrp affect intestinal secretion of urate or not. In this report, we examined whether indoxyl sulfate (IS) is involved in the upregulation of BCRP in intestine at CKD state. After exposure to IS, mRNA level, and protein level of BCRP in Caco-2 cells were assayed by real-time PCR and flow cytometry, respectively. BCRP mRNA level was increased by exposure to IS for 24 h at a concentration dependent manner and reach steady state at 0.2 mM, a clinical relevant concentration. BCRP expression in membrane fraction was also increased by 1.8 folds after treatment with 0.2 mM for three days. Basolateral-to-apical transport of urate in Caco-2 cells had 22% increase after IS treatment. Intracellular accumulation of selective substrate of BCRP, pheophorbide a, was also decreased by 22% after IS treatment. IS was also observed to increase BCRP mRNA expression in LS180 cells and HepG2 cells, which are enterocytes- and hepatocytes-model cell lines, respectively. However, no protein was expressed in membrane fraction of LS180 cells. BCRP protein expression in membrane fraction of HepG2 cells was increased after IS treatment, whereas no function of BCRP was observed in HepG2 cells. Results indicate that indoxyl sulfate might be involved in the up-regulation of BCRP at CKD state.

4-1 Introduction

Urate is the final product of purine metabolism in humans. Due to the loss of uricase in the evolution of human being, urate cannot be degraded into allantoin which is more soluble than urate. In humans, urate primarily excretes through kidney and intestine. Traditionally, kidney is regarded as an important organ in the excretion of urate. It has been revealed that almost two thirds of urate daily produced is excreted through kidney [125-126]. In many cases, reduced function of kidney has been associated with gout and hyperuricemia [127-129]. However, the rise of serum urate level is not high compared with many other solutes which may be excreted by kidney at chronic kidney failure state, a state during which renal function is greatly reduced. This might be explained by a compensatory increase of intestinal urate excretion, as is reported by Vaziri et al. who observed the increase of intestinal urate excretion in CRF rats [130]. Recent genome-wide association studies show that breast cancer resistance protein (BCRP, encoded by ABCG2), which is highly expressed in intestine, is associated with the cause of gout and hyperuricemia [131-137]. Urate was identified as the substrate of BCRP by efflux experiments using oocytes expressing ABCG2 gene and decreased intestinal urate excretion was observed in Bcrp knockout mice [138]. Yano et al. reported that BCRP expression was increased in the intestine of CRF rats [139].

Indoxyl sulfate (IS), a derivative of diary protein, is generated in intestine by bacteria-mediated protein-derived tryptophan metabolism and mainly excreted by kidney [140-141]. Members from OAT family, OAT1 and OAT3, located at renal proximal tubule are responsible for the excretion of IS [142-146]. At normal state, serum indoxyl sulfate concentration is about 2.5 μM in humans [147-148]. In patients with chronic kidney failure, serum indoxyl sulfate concentration can be increased to as much as 210 μM [147-148]. Indoxyl sulfate exhibits numerous biological functions. It induces oxidative stress in

many kinds of cells, inhibits NO production, and has an inhibitory effect on endothelial proliferation [149-153]. It also stimulates glomerular sclerosis and plays a pivotal role in the progression of kidney failure [154-155]. Recent reports indicate that indoxyl sulfate exhibits as a potent endogenous agonist for the aryl hydrocarbon receptor (AhR) [156], a transcriptional activator of BCRP [157]. In the present study, we investigated whether indoxyl sulfate is involved in the compensatory increased intestinal urate excretion at CKD state.

4-2 Materials and Methods

4-2-1 Chemicals and reagents

Indoxyl sulfate (potassium salt) was purchased from Nacalai Tesque (Kyoto, Japan). 3-Methylchoranthrene (3-MC) and Ko143 were from Sigma-Aldrich (St. Louis, MO). Pheophorbide a (PhA) and albumin from bovine serum (Fraction V) were the products of Wako Pure Chemical Industries (Osaka, Japan). Fluorescein isothiocyanate (FITC) labeled 5D3 antibody (anti-BCRP) and FITC-labeled isotype control (mouse IgG2b) were purchased from BioLegend (San Diego, CA). [^{14}C]Urate (1.96 TBq/mol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). All other reagents were of analytical grade.

4-2-2 Cell culture

Caco-2 cells, HepG2 cells and LS180 cells obtained from the American Type Culture Collection (Rockville, MD) were cultured at 37 ^{o}C in Dulbecco' s modified Eagle's medium (D-MEM; Wako Pure Chemical Industries, Osaka, Japan) with L-Glutamine and phenol red, supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Thermo Scientific, Logan, UT, USA), 1% (v/v) MEM nonessential amino acids (Wako Pure Chemical Industries, Osaka, Japan), 100 units/mL penicillin, and 100 μg/mL streptomycin.

4-2-3 Total RNA isolation and real time PCR

Total RNA from the cells was extracted by the addition of Isogen (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's operational manual. Briefly, after the removing the culture medium, cells were washed by pH7.4 phosphate buffered saline (PBS) twice. Isogen was then added into each well and samples of each well were transferred into a 1.5 mL tube after 5 min. Chloroform was added to purify RNA and isopropanol was used to precipitate RNA. The concentration of total RNA was determined by UV method on an Eppeendorf BioPhotometer (Eppendorf, Hamburg,

Germany). 1 μg of total RNA was used for the synthesis of cDNAs with a High Capacity cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA). RNA was mixed with 2 μL 10xRT Random Primers supplemented with purified water to 10 μL and was denatured at 65 ^{o}C for 7min.

Reverse transcription reactions were prepared by mixing denatured RNA with following components:

10xRT buffer	2.0 μL
100 mM dNTP Mix	0.8 μL
Reverse Transcriptase	1.0 μL
Pure water	6.2 μL
Total:	10 μL

Reverse transcription was then carried out on GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) using following program:

Step1: 25°C 10min

Step2: 37°C 120min

Step3: 85°C 5 s

Step4: 4°C ∞

Real-time PCR

For real-time PCR reaction was performed using a Stratagene Mx3000P real-time PCR system (Agilent Technologies, La Jolla, CA, USA). Master Mix was made by following method:

primer mix (each 10 μM)	0.75 μL
2 X SYBRGS	7.5 μL
ROX dye	0.2 μL
purified water	5.55 μL
total	14 μL

Then 14 μL of Master Mix was transferred into a reaction tube. 1μL of reverse transcription product was added into the reaction tube and mixed.

PCR reaction was cycled as follows:

95°C 10min x1

94°C (10s)
55°C (10s) x45 cycles
72°C (15s)

Cool to 4°C

1 μL of the reverse transcription product was mixed with 0.75 μL of 10 μM primer mix, 0.2 μL ROX reference dye (Agilent Technologies), 5.55 μL of purified water and 7.5 μL of 2X Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix (Agilent Technologies) to perform the real-time PCR using a Stratagene Mx3000P real-time PCR system (Agilent Technologies, La Jolla, CA, USA). The PCR circling conditions were as follows: denaturation at 95 ^{o}C for 10 min; 45 cycles of 94 °C for 10 s, 55 °C for 10 s, 72 °C for 10 s; and 1 cycle of 95 ^{o}C for 1 min, 55 ^{o}C for 30 s, 95 ^{o}C for 30 s.

Data were analyzed by MxPro QPCR software (Version 4.10, Stratagene). ΔΔCt method was used to calculate the relative gene expression of BCRP and the expression of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as the internal control. Sequences of the primers used for BCRP and HPRT detection was as follows. For BCRP (product size 187 bp): 5′-GTTTCAGCCGTGGAAC-3′ (sense); 5′-CTGCCTTTGGCTTCAAT-3′ (antisense). For HRPT (product size 94 bp): 5′-TGACACTGGCAAAACAATGCA-3′ (sense); 5′-GGTCCTTTTCACCAGCAAGCT-3′ (antisense).

4-2-4 Expression of BCRP in membrane fraction

Expression of BCRP in membrane fraction was examined by flow cytometer on a BD FACScan™ flow cytometry system (BD Biosciences, Mountain View, CA). Cells were trypsinized by 0.1% trypsin (BD Biosciences, Sparks, MD) at room temperature for up to 5 mins. Cell pallet was obtained by centrifuging at 200 g

for 5min. Cells were resuspended in 2% BSA/PBS and kept on ice for 30 min. After that, cells were incubated with FITC-labeled 5D3 antibody or FITC-labeled isotype control (mouse IgG2b) at 14°C for 3h in the dark at the concentration of 1:10 in 2% BSA/PBS solution. After immunofluorescent staining, cells were washed by PBS and kept in dark on ice until analysis. Surface expression of BCRP was calculated by comparing the fluorescence intensity (Geo mean value) of the cells stained by 5D3 and the isotype control.

4-2-5 Intracellular accumulation of PhA in Caco-2 cells

Intracellular accumulation of PhA in Caco-2 cells was examined by flow cytometeron a BD FACScan™ flow cytometry system (BD Biosciences, Mountain View, CA). Caco-2 cells totally cultured for two weeks were trypsinized by 0.1% trypsin at room temperature for up to 5 min. Cell pallet was obtained by centrifuging at 200 g for 5 min. Cells were re-suspended in culture medium containing 5 μM PhA with or without 1 μM Ko143 and incubated at 37°C for 60 min in dark. After that, cells were centrifuged at 200g for 5 min to get the pellet. The resultant cell pellet was washed by 1 mL PBS and re-suspended in 0.5 mL PBS. Samples were kept in dark on ice and subsequently analyzed by flow cytometry. Intracellular accumulation of PhA was calculated by comparing the fluorescence intensity (Geo mean value) of the cells with or without the addition of Ko143 during the uptake.

4-2-6 Transcellular urate transport in Caco-2 cells

To investigate whether IS treatment has any effect on the transcellular urate transport in Caco-2 cells, Caco-2 cells were treated for 3 days with 0.2 mM IS on both apical side and basolateral side before the experiment, totally cultured for three weeks on Transwell filter membrane inserts (BD Falcon, surface area 0.9 cm^2 and pore size 3 mm) after subculture at a density of $6.4X10^{-4}$/well. Then, transcellular urate transport study was carried out according to the method previously described [52]. The apparent permeability (P_{app}, cm/sec) of

[^{14}C]urate across the cell monolayer from basolateral side to apical side was calculated by following equation:

$$P_{app} = \frac{dQ}{dt} * \frac{1}{AC0}$$

In which Q is the amount of [^{14}C]urate transported over time t, C_0 is the initial concentration in the basolateral side and A is the surface area of membrane.

4-3 Results

4-3-1 mRNA expression of BCRP in Caco-2 cells

mRNA expression of BCRP in Caco-2 cells was firstly examined by real-time PCR. Caco-2 cells were seeded on a 24 well plate at a density of $1x10^5$ cells/well and were exposed to 1.0 mM indoxyl sulfate for 24 h before the experiment (totally cultured for 2 weeks) after subculture. Ten μM 3-MC was used as positive control for induction of BCRP. mRNA expression of BCRP in Caco-2 cells was increased by approximately 4 fold after the exposure to indoxyl sulfate and 10 μM 3-MC (**Fig. 4-1A**). IS also showed an induction of expression of Bcrp mRNA in a concentration dependent manner (**Fig. 4-1B**). It reached steady state at 0.2 mM which was clinically relevant concentration at disease state. Thus, 0.2 mM of IS treatment was used as further studies.

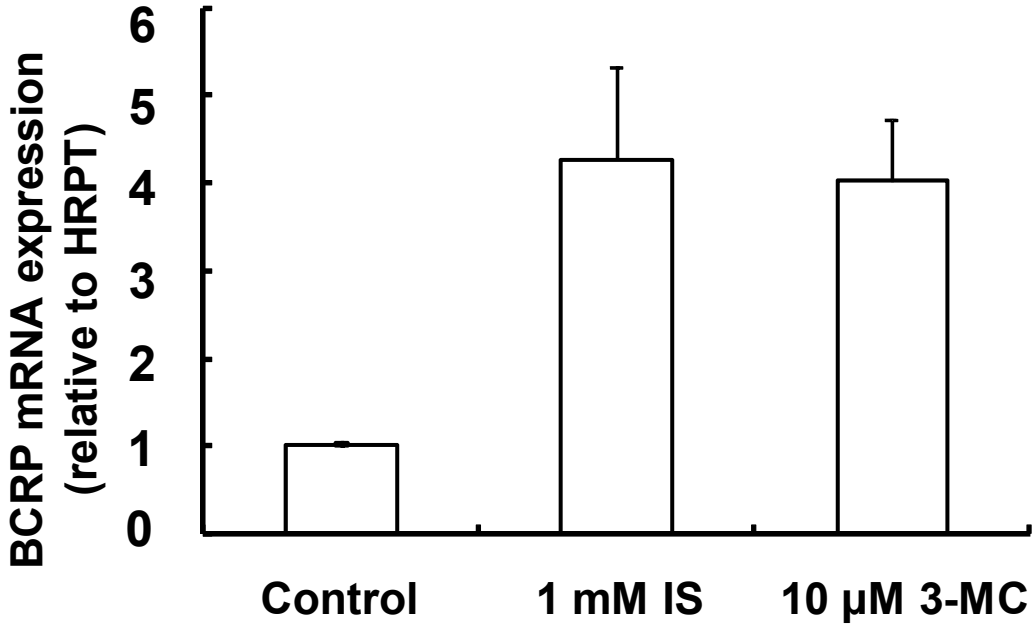
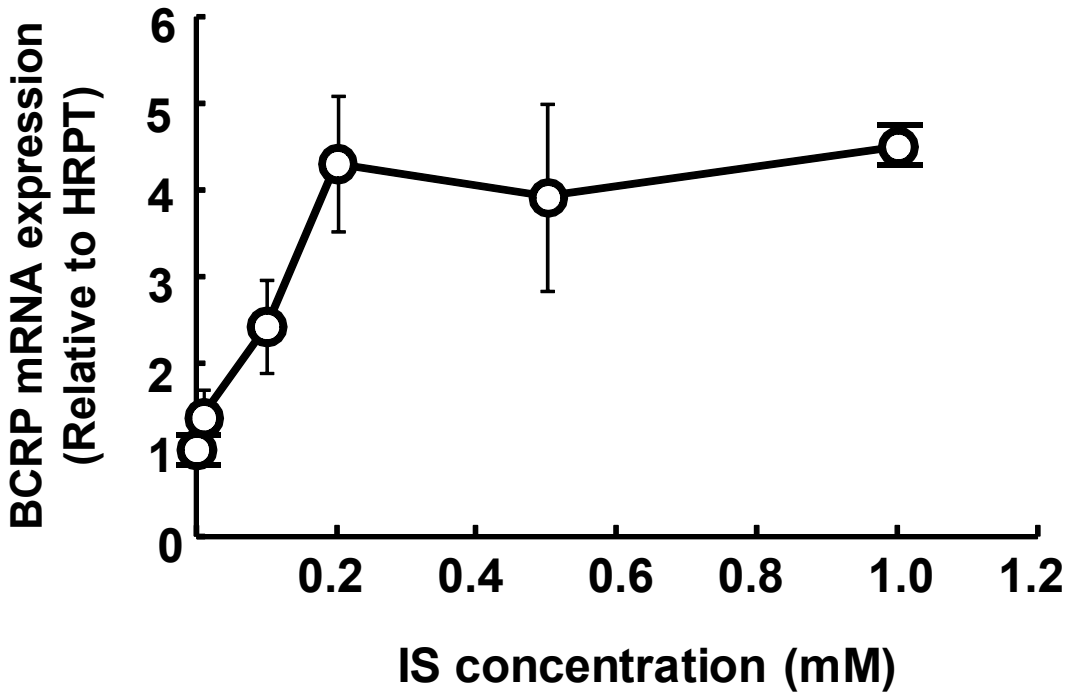


Fig. 4-1 mRNA level of BCRP in Caco-2 cells treated with IS. (A) After 24h exposure, BCRP mRNA level was increased approximately 4 folds by IS and 3-MC. Data represent as mean ± SEM (n=3).



(B) After 24h exposure, BCRP mRNA level was increased at a concentration dependent manner by 0.01-1 mM IS. Data represent as mean± SEM (n=3).

4-3-2 Protein expression of BCRP in membrane fraction of Caco-2 cells

Caco-2 cells were seeded on a 24 well plate at a density of $1x10^5$ cells/well and were exposed to 0.2 mM indoxyl sulfate for 1, 3, and 5 days before the experiment, with totally cultured for two weeks after subculture. BCRP expression in membrane fraction of Caco-2 cells was subsequently examined using flow cytometry method with FITC-conjugated 5D3 as the antibody against BCRP protein. Expression of BCRP in membrane fraction of Caco-2 cells showed a time-dependent incresae after treatment with 0.2 mM. After three days' exposure, BCRP expression reached steady state by increasing 1.8 folds compared with non-treated control group (**Fig. 4-2**). Longer exposure time could not result in higher BCRP expression at membrane fraction.

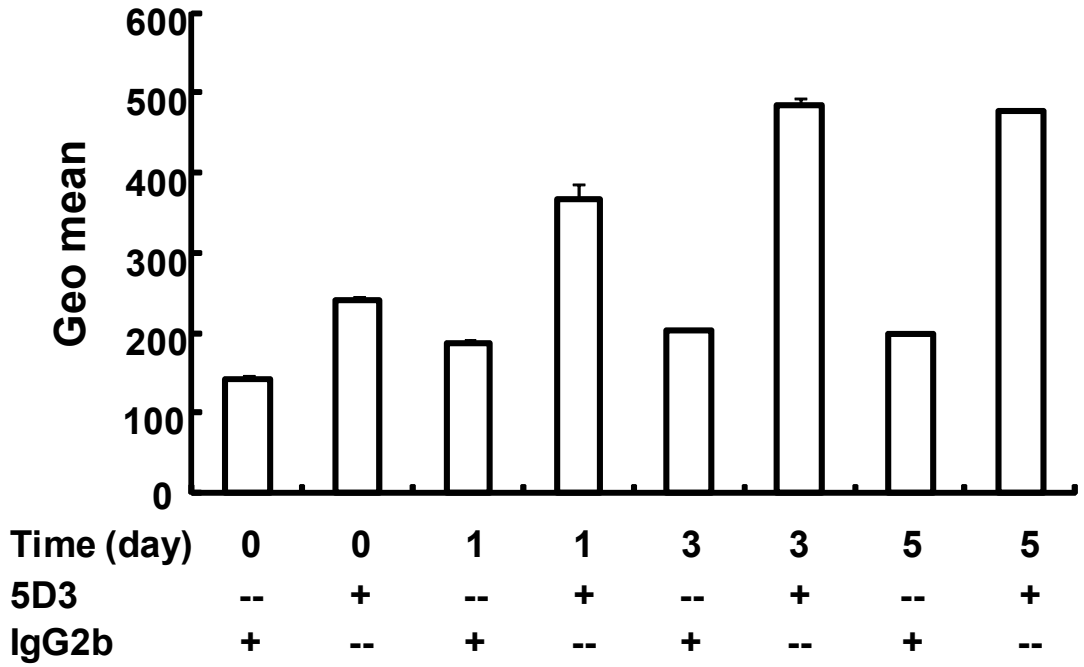


Fig. 4-2 Time dependent increase of BCRP protein after 0.2 mM IS treatment. FITC-conjugated 5D3 was used as the antibody against BCRP protein. FITC-conjugated mouse IgG2b was used as isotype control. After treated for three days, BCRP expression in membrane fraction reached steady state. Data represent as mean ± SEM (n=3).

4-3-3 Transcellular urate transport in Caco-2 cells treated with IS

Transcellular urate transport was carried out to investigate whether IS treatment has any effect on the function of cells. After exposure the cells to 0.2 mM IS for three days before the transport study, urate transport increased by 22%, compared with the non-treated control group (**Fig. 4-3**).

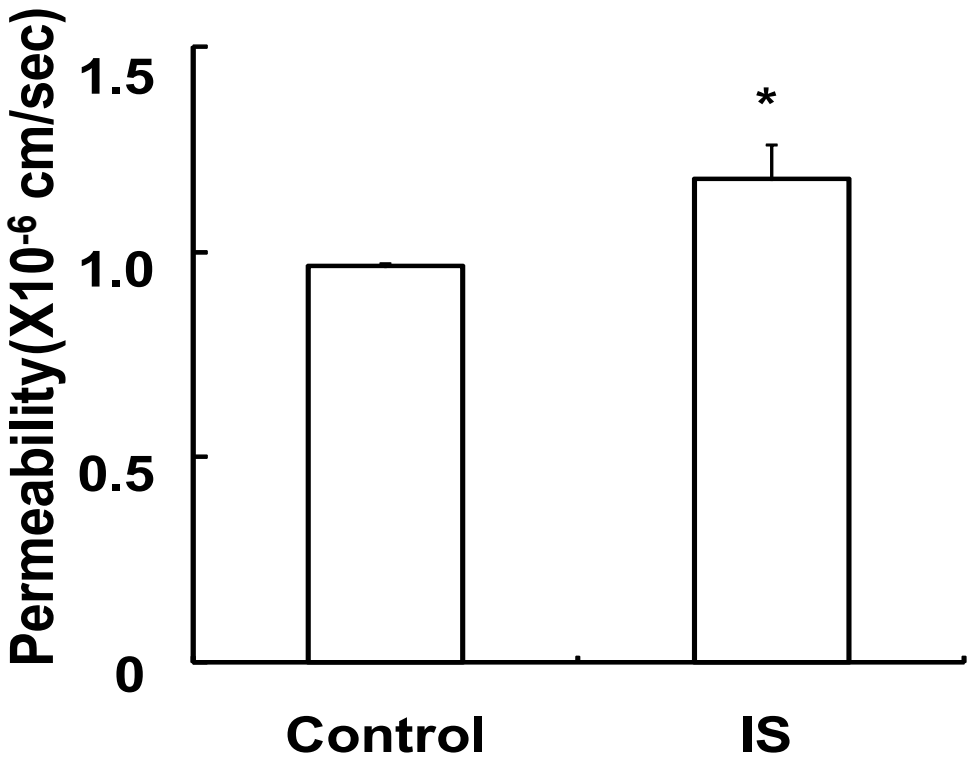


Fig. 4-3 Transcellular transport of urate in Caco-2 cells. After treated with 0.2 mM IS for three days, permeability of urate in Caco-2 cells has 22% increase compared with that of the non-treated control group. Data represent as mean ± SEM (n=3). Student's t-test: *, $p<0.05$.

4-3-4 Intracellular accumulation of PhA in Caco-2 cells after IS treatment

To investigate whether mRNA and protein increase of BCRP can result in any functional increase of BCRP, intracellular accumulation of PhA, a typical BCRP substrate, was studied by flow cytometry method. Function of BCRP in Caco-2 cells cultured for two weeks was observed as shown in **Fig.4-4**, due to the reduced intracellular accumulation of PhA in the absence of 1 μM Ko143. In control group, intracellular accumulation of PhA was 88.7% of that in the presence of Ko143. After IS treatment, intracellular accumulation of PhA decreased from 88.7% to 66.7% of that in the presence of Ko143, which indicated that function of BCRP was increased after IS treatment.

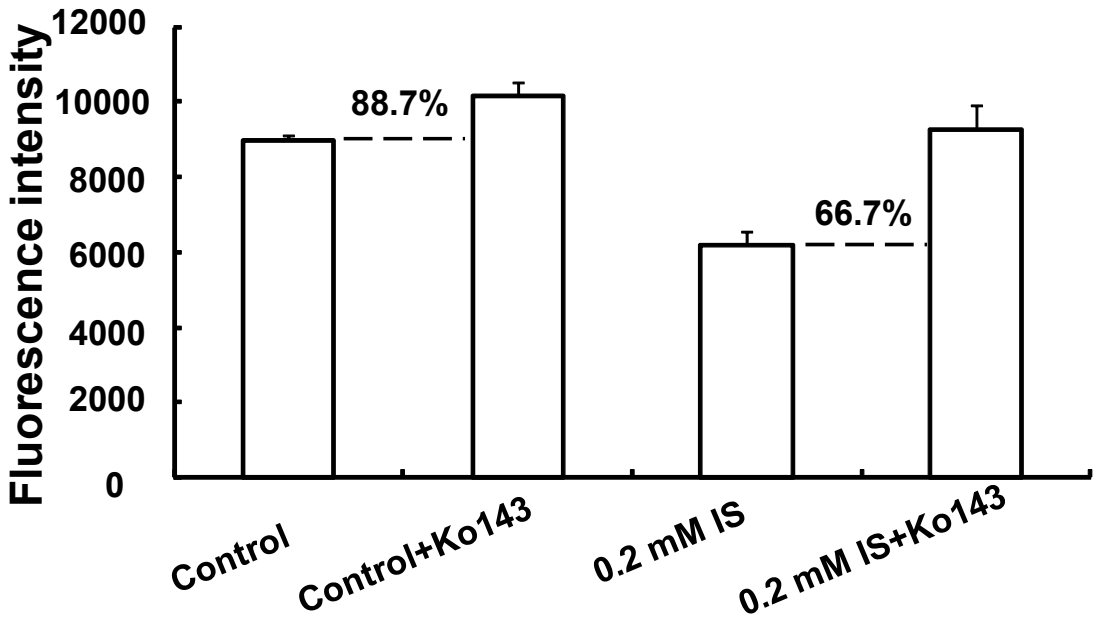


Fig. 4-4 Intracellular accumulation of PhA in Caco-2 cells. Caco-2 cells were treated with IS for 3 days before the experiment (totally cultured for two weeks). Intracellular accumulation of PhA reduced to from 88.7% to 66.7% compared with the cells in the presence of 1 μM Ko143. Data represent as mean ± SEM (n=3).

4-3-5 Effect of IS on BCRP mRNA expression in LS180 cells

We further examined whether the induction of BCRP by IS exists in other intestinal cell lines. LS180 cells were exposed to IS at concentrations from 0.1-1mM for 48 hours after subcultured on a 24 well plate at a density of $1x10^5$ cells/well. Total RNA was subsequently extracted and mRNA level of BCRP was examined by real-time PCR. IS showed an increase in BCRP mRNA level in a concentration-dependent manner. After treatment with 0.2 mM IS for 48 hours, BCRP mRNA expression was increased by 122 folds (**Fig. 4-5**).

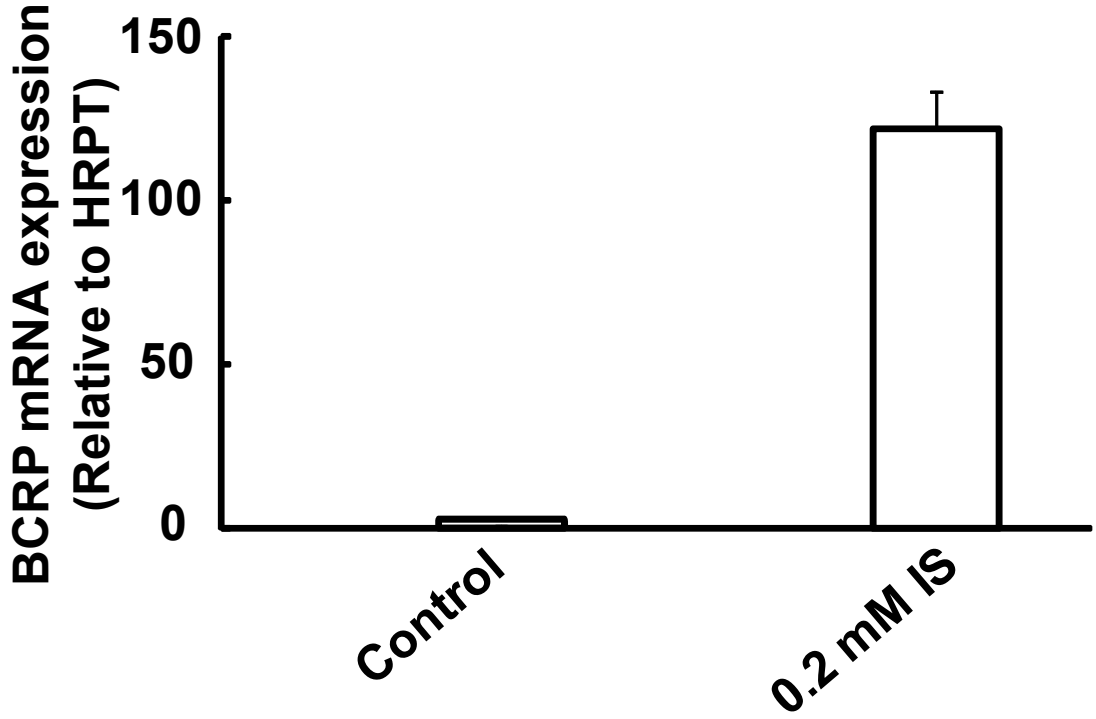


Fig. 4-5 Effect of IS on BCRP mRNA expression in LS180 cells. LS180 cells were treated with 0.2 mM IS for 48h after subculture. BCRP mRNA expression was increased by 122 folds. Data represent as mean ± SEM (n=3).

4-3-6 Protein expression of BCRP in membrane fraction of LS180 cells

BCPR expression in membrane fraction of LS180 cells was examined after treatment with 0.2 mM IS for 48 h after subculture at a density of $1x10^5$ cells/well in a 24-well plate. BCRP expression in membrane fraction of LS180 cells was subsequently examined using flow cytometry method with FITC-conjugated 5D3 as the antibody against BCRP protein and with FITC-conjugated mouse IgG2b as isotype control. Expression of BCRP in membrane fraction of LS180 cells has no significant change after IS treatment (**Fig. 4-6**).

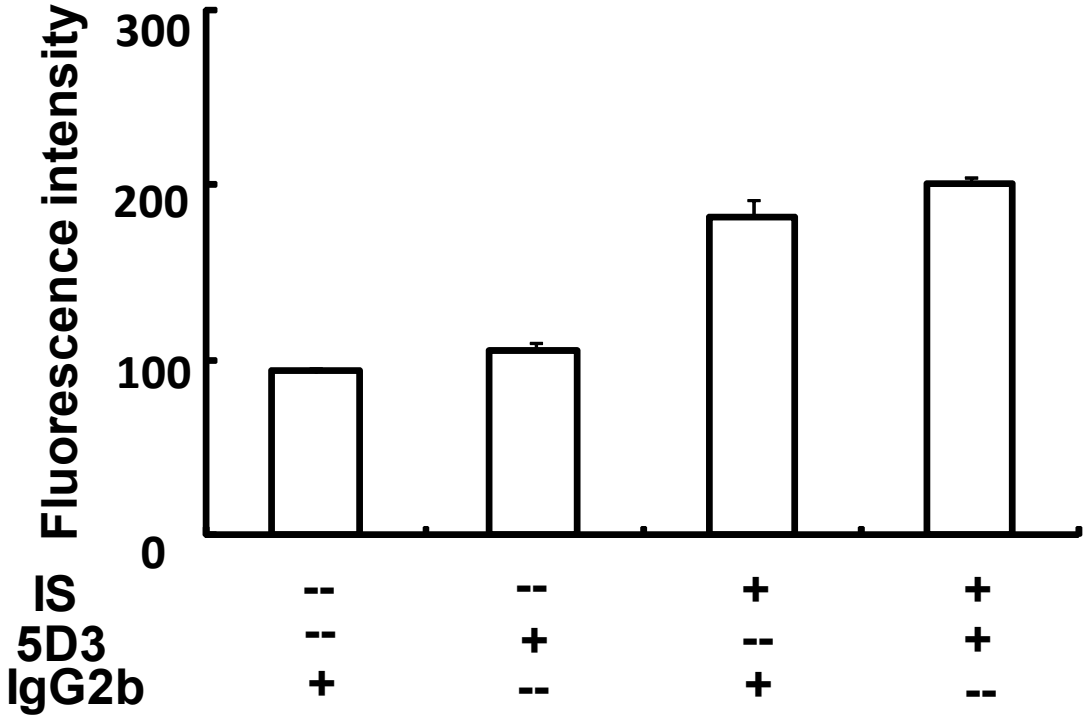


Fig. 4-6 Protein expression of BCRP in membrane fraction of LS180 cells. LS180 cells was examined after treatment with 0.2 mM IS for 48 h and BCRP protein expression in membrane fraction was subsequently examined by flow cytometry after staining with FITC-conjugated 5D3 antibody. FITC-conjugated mouse IgG2b was used as isotype control. No significant change of membrane BCRP protein expression was observed after IS treatment. Data represent as mean ± SEM (n=3).

4-3-7 Effect of IS on BCRP mRNA expression in HepG-2 cells

Because BCRP is also reported to be expressed in liver which might be involved in non-renal urate excretion, we also examined whether IS has any effect on BCRP expression in liver cells. HepG2 cells were exposed to IS at concentrations from 0.1-1 mM for 24 h after subculture on a 24 well plate at a density of $1x10^5$ cells/well for 7 h. BCRP mRNA level was subsequently examined. BCRP mRNA level in HepG2 cells was increased in a concentration dependent manner both for IS and 3-MC induction (**Fig. 4-7**).

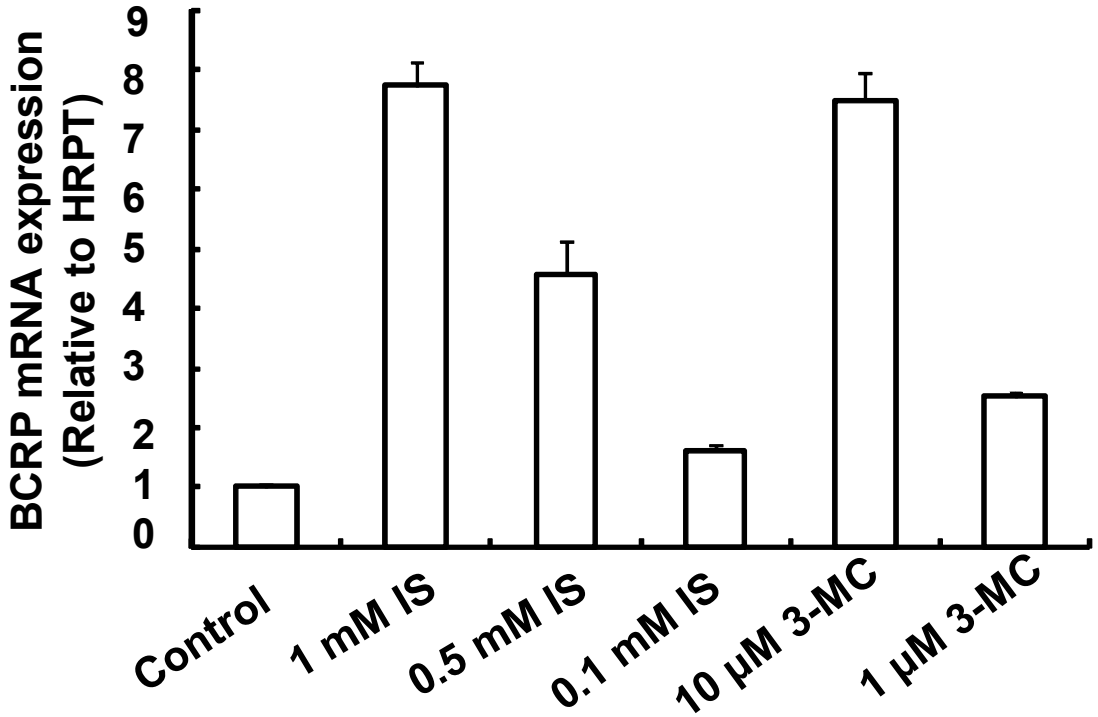


Fig. 4-7 Effect of IS on mRNA expression of BCRP in HepG-2 cells. After 24 h IS treatment, BCRP mRNA level in HepG2 cells was increased in a concentration dependent manner from 0.1 to 1 mM. 3-MC also increased BCRP mRNA level in a concentration dependent manner from 1 to 10 μM. Data represent as mean ± SEM (n=3).

4-3-8 Protein expression of BCRP in membrane fraction of HepG2 cells

BCPR expression in membrane fraction of HepG2 cells was examined after treatment with 0.2 mM IS for 48 h after subculture at a density of $1x10^5$ cells/well in a 24-well plate. BCRP expression in membrane fraction of HepG2 cells was subsequently examined using flow cytometry method with FITC-conjugated 5D3 as the antibody and FITC-conjugated mouse IgG2b was used as isotype control. Expression of BCRP in membrane fraction of HepG2 cells has 3-fold increase after IS treatment (**Fig. 4-8**).

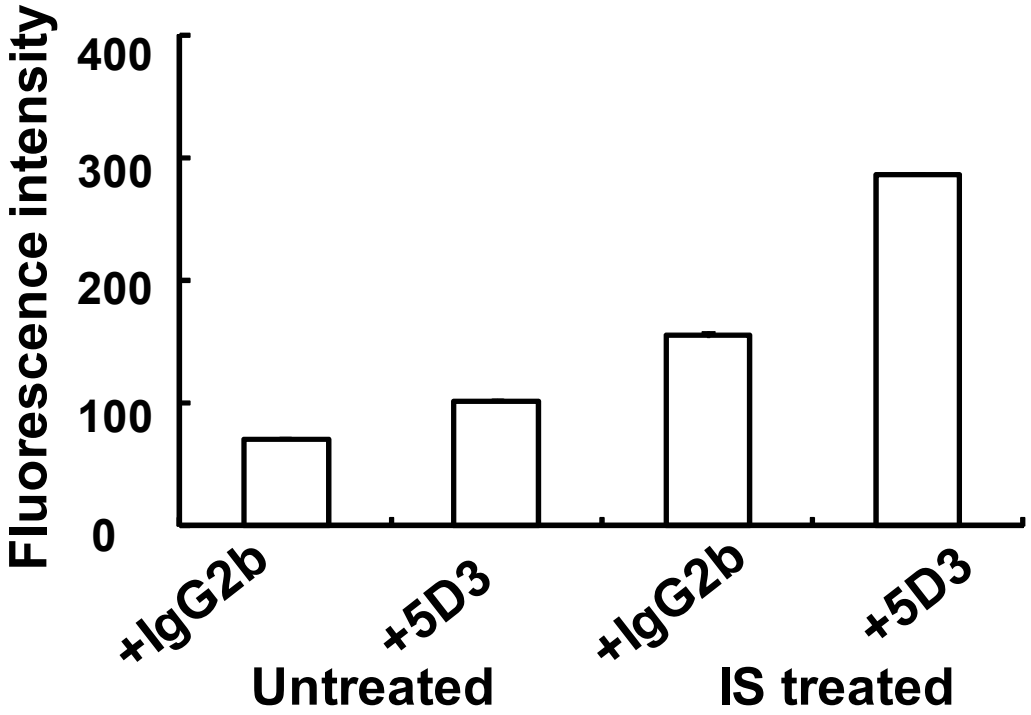


Fig. 4-8 Protein expression of BCRP in membrane fraction of HepG2 cells. BCRP expression in membrane fraction of HepG2 cells was examined after treatment with 0.2 mM IS for 48 h and BCRP protein expression in membrane fraction was subsequently examined by flow cytometry after staining with FITC-conjugated 5D3 antibody. FITC-conjugated mouse IgG2b was used as isotype control. After IS treatment, BCRP membrane expression had 3-fold increase. Data represent as mean ± SEM (n=3).

4-3-9 Intracellular accumulation of PhA in HepG2 cells treated with IS

Due to the increase of membrane BCRP protein observed in HepG2 cells after IS treatment, we next investigated whether mRNA and protein increase of BCRP can result in any functional increase of BCRP in HepG2 cells. Intracellular accumulation of PhA was studied by flow cytometry method. Ko143 seems to have no effect on intracellular accumulation of PhA in control cells (**Fig. 4-9**). Similar phenomenon was observed in cells after IS treatment (**Fig. 4-9**). This indicated that BCRP expression might be too low to show some function in HepG2 cells.

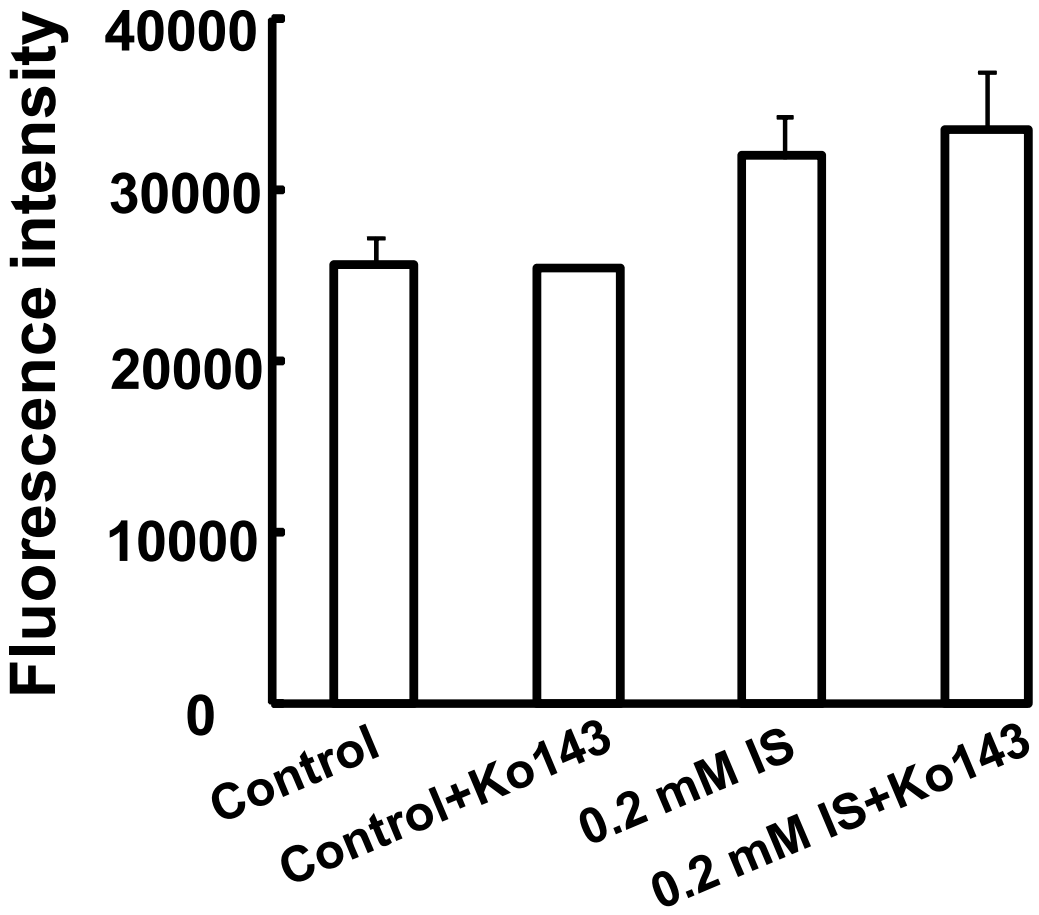


Fig. 4-9 Intracellular accumulation of PhA in HepG2 cells after IS treatment. HepG2 cells was examined after treatment with 0.2 mM IS for 48 h and Intracellular accumulation of PhA was subsequently examined by flow cytometry after incubation with 5 μM PhA for 60 min. Data represent as mean ± SEM (n=3).

4-4 Discussion

Past decades have witnessed a great increase in the prevalence of CKD. The cost used for the treatment of CKD has been steadily increasing throughout the world. To investigate the physiological change at CKD state is crucial for the better understanding of the mechanism of the disease, providing essential knowledge for the treatment of CKD. It has been reported that intestinal urate excretion was increased at CKD state. In the present study, we depicted the possible role of IS, a well-recognized uremic toxin, in the induction of BCRP at CKD state as a compensation of the body to the decreased renal function.

Initially, Caco-2 cells were selected as an intestinal epithelial model cell line to examine the effect of IS on BCRP expression. Both mRNA level and membrane protein level of BCRP were increased after treatment of IS at a clinically relevant concentration. We next investigated the functional change of BCRP after IS treatment in Caco-2 cells. Accumulation of PhA, a probe compound for the evaluation of BCRP function, was decreased after IS treatment. Similarly, basolateral to apical directed urate transport was increased by 22% after IS treatment (**Fig. 4-3**). The increase of urate transport after exposure to IS was relatively small (22%) compared with the increase in mRNA level (3 folds) and membrane fraction protein expression (1.8 folds). This might be attributed to several reasons. At first, transporters expressed at the basolateral side of Caco-2 cells may not be efficient in transporting urate from basolateral side into Caco-2 cells. Secondly, influx transporters on the apical side may be also upregulated by IS, reducing net urate efflux by Caco-2 cells. Or other transporters than BCRP, that is not affected by IS is also contributing to this transport.

In LS180 cells, BCRP mRNA level was quite low as is reported by previous study, over 100-fold lower than that in Caco-2 cells [158]. After IS treatment,

BCRP mRNA level was increased by 122 fold as is shown in **Fig.4-5**. However, no protein expression was detected in membrane fraction of LS180 cells before and after IS treatment. This might be due to the lack of other transcriptional factors for BCRP protein expression in LS180 cells.

In HepG2 cells, both BCRP mRNA level and membrane protein expression were increased after IS treatment (**Fig. 4-7** and **Fig. 4-8**). However, no functional increase was observed (**Fig. 4-9**). BCRP seems to have no function in HepG2 cells. Because Ko143 could not increase the intracellular accumulation of PhA in HepG2 cells. BCRP protein expression in HepG2 cells might be too low and it is difficult to detect its function under current experimental conditions.

BCRP expression can be regulated by many factors. For example, AhR has shown the inductive effect on BCRP expression in human intestinal, liver, and mammary carcinoma cells and in primary colonocytes and hepatocytes [157]. Also, peroxisome proliferator-activated receptor-α agonist can up-regulate the expression of Bcrp in mice intestine [159]. IS might stimulate AhR to up-regulate BCRP expression, because it has been reported as a potent AhR agonist [156]. In the present study, 3-MC, an AhR agonist was used as positive control and it exhibited up-regulatory effect on BCRP expression (**Fig. 4-1A** and **Fig. 4-7**). This indicated that stimulation of AhR might be involved in the upregulation of BCRP by IS. However, other stimulation factors than AhR might also exist. Thus, further studies are still needed to illustrate the mechanism of the phenomenon observed in the present study.

Results from the present study show that BCRP expression and transcellular urate transport can be enhanced by IS at clinically relevant concentration. IS might be involved in the upregulation of intestinal BCRP expression at CKD

state and responsible for the compensatory increase of non-renal urate excretion.

Chapter 5 Conclusion

Since the discovery of URAT1 in 2002, many compounds which can lower serum urate level have been identified as URAT1 inhibitors. These compounds can influence serum urate level in a direct manner by blocking URAT1 directly. In Chapter 2, a cooperative relationship of SMCT1 and URAT1 was proved. This indicates that SMCT1 is also involved in renal urate transport, but it works in an indirect manner in the regulation of serum urate level. Thus, SMCT1 is possibly to be used as a new target for the development of novel compounds to regulate serum urate level in an indirect manner. This kind of compounds to be developed might be used alone or in combination with other URAT1 inhibitors to regulate serum urate level, increasing the efficiency of current compounds or reducing the side effect of current compounds.

In Chapter 3, interaction of whisky congeners with urate transporters was studied as a new rationale for their effect on lowering serum urate level. Inhibition of URAT1 by congeners might be involved in the lowered serum urate level after whisky consumption. Due to the complexity of whisky congeners, further studies are still needed to identify what kind of compounds are more likely to be responsible for the inhibition of URAT1.

Chapter 4 focused on efflux urate transporter and investigated the change of BCRP at disease state. It shows that indoxyl sulfate, a model uremic toxin, might be involved in the regulation of BCRP and contributes to the increased compensatory non-renal urate excretion. The molecular mechanism of this phenomenon still needs to be investigated.

In conclusion, this thesis shows that serum urate level can be regulated by directly or indirectly influencing urate transporters and humans have a self-compensatory mechanism to regulate serum urate level at disease state

by modulation of urate transporters. The results of this thesis indicate that transporters involved in urate transport can be used as targets for developing novel compounds to control serum urate level.

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1. Y. Lu, T. Nakanishi, I. Tamai. Functional cooperation of SMCTs and URAT1 for renal reabsorption transport of urate. Drug Metab Pharmacokinet. 28: 153-158 (2013).
2. Y. Lu, T. Nakanishi, M. Fukazawa, I. Tamai. How does whisky lower serum urate level? Phytother Res. (in Revision).

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